THE CULTIVATION OF TRYPANOSOMES IN ARTHROPOD TISSUE CULTURE

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SUMMARY

The literature relating to the development of the technique of arthropod tissue culture, its application to biological research, and the cultivation of trypanosomes is reviewed.

Cultivation of tissues and organs from Glossina morsitans morsitans was investigated using several culture media. Of the media tested, modified Trager's medium was found to be the best for the growth and maintenance of tissues. Successful cultures of developing adult tissues from ticks, Rhipicephalus appendiculatus, R.bursa and Amblyomma hebraeum and from partially engorged adult Ixodes ricinus were obtained in VP_4 medium.

The greatest success in the cultivation of Trypanosoma brucei and T.congolense in association with Glossina tissues was achieved in the presence of complete alimentary tract of pupae older than 21 days in 15-20µl hanging-drops of modified Trager's medium incubated at 28°C. Growth of the trypanosomes also occurred in cultures in which the flagellates were separated from the insect tissues by a semi-permeable membrane. No multiplication of the parasites was observed in (a) culture medium alone, (b) medium containing extracts of alimentary tract and (c) medium in which alimentary canal had been cultured for 3 or 4 days.

In all the cultures in which multiplication of the trypanosomes occurred, it was accompanied by morphological transformation. Bloodstream trypomastigotes in the inoculum changed into long slender forms similar to those seen in the midgut of the tsetse fly. Infective stages were not observed.

Complete digestive tract from G.morsitans submorsitans,

G.austeni and G.palpalis was as suitable as that from G.m.morsitans for the cultivation of the trypanosomes and the same applied to the alimentary tract of the non-haematophagous fly Sarcophaga, a dipteran closely related to Glossina. On the other hand, cultures of comparable tissues from mosquitoes, Aedes aegypti and ticks, R.appendiculatus failed to support the growth of trypanosomes.

Fly transmissible as well as laboratory adapted strains of T.brucei and T.congolense could be cultivated in tsetse organ cultures. Negative results were obtained, however, with laboratory adapted strains of T.gambiense and T.vivax and with the monomorphic T.evansi which is not cyclically transmitted in nature by tsetse flies.

Stercorarian species, Trypanosoma musculi, T.lewisi, T.melophagium and T.theileri were also grown in the presence of tsetse alimentary tract. All these species transformed into and multiplied in forms resembling those formed in the hindgut and rectum of their arthropod vectors. Unlike the cultures of the salivarian trypanosomes, those of T.musculi and T.lewisi remained infective to laboratory rodents for many days.

Amino acid analyses of haemolymph of pupae, pre-emerged and adult G.morsitans revealed that of the 21 amino acids, proline was present in highest concentrations in all the stages examined. There were also considerable differences in the concentrations of the individual amino acids in these three stages. The results of the amino acid analyses formed bases for the design of culture media which were capable of supporting growth of T.brucei and T.congolense.

CONTENTS

Chapter 1

INTRODUCTION

Many pathogenic protozoa, bacteria and viruses parasitic in man and domestic animals undergo part of their development in arthropod hosts. The requirements of some of these parasites are so exacting that they cannot be maintained *in vitro* except in the presence of host cells. Since the methods for culturing vertebrate tissues and cells have been well established, it is not surprising that most attention has been given to the developmental stages of the parasites which occur in the vertebrate hosts. Far less has been accomplished with regard to the parts of the cycles limited to the arthropod vectors. However, recent improvements in the methods of cultivation of arthropod tissues and cells have made possible studies of the stages developing in the vectors. This is reflected clearly in the numerous reports on the *in vitro* development of the mosquito-borne stages of arboviruses and malaria parasites.

It is somewhat surprising that the cultivation of tissues from ticks and tsetse flies has not received more attention during recent years. The former are known to be the vectors of pathogenic protozoa, rickettsiae and viruses highly pathogenic to man and domestic animals, and the latter transmit salivarian trypanosomes. In the field of protozoology, by the use of tsetse tissue cultures, much can be learned about the developmental stages of the African pathogenic trypanosomes and similar cultures of ticks would also allow us to gain some insight into the development of protozoa transmitted by ticks. Indeed, a survey of the available literature

provides convincing evidence that, to date, relatively little is known about the invertebrate phases of these parasites. In addition to providing a useful tool for parasitological research further development and refinement of methods for the cultivation of arthropod tissues should also prove useful in studying the structrual and functional aspects of these tissues in various stages of their development.

One of the aims of the research detailed in this thesis was to devise methods of cultivation of tsetse fly and tick tissues which would provide systems suitable for studies of the developmental stages of the protozoan parasites which are transmitted by these vectors. Since quite early in this century tissue culturists have tried to solve the various problems of inducing arthropod cell proliferation and maintaining healthy arthropod tissues *in vitro*. For 50 years the difficulties appeared almost insurmountable but about a decade ago many of them were resolved and the cultivation of arthropod cells and tissues became a reality. The successes achieved depended a great deal upon the accumulation in the late 1950's and the early 1960's of data on the composition of insect blood. More recently studies by Cunningham and Slater (1974) which provided information about the amino acid composition of tsetse haemolymph have contributed significantly to this data.

The principal stimulus for the investigations reported here was the work of Trager (1959), who succeeded in obtaining extensive cell migration from primary explants of various tissues of *Glossina palpalis*. Although, somewhat less spectacular the results of Nicoli and Vattier (1964) with *in vitro* maintenance

of Glossina fuscipes tissues added to the interest in the opportunities provided by the tsetse tissue culture system in the studies of the development of salivarian trypanosomes.

The work reported in this thesis was, therefore, directed towards:

(1) finding suitable donors of the explants in terms of age and state of differentiation of the tsetse pharate flies and developing adult ticks,

(2) devising a medium capable of supporting proliferation of the donor cells and

(3) providing a system which would favour both the arthropod tissues and the parasitic protozoa

While the majority of the experiments described involved the tissues of tsetse flies and the trypanosomes associated with them, investigations were extended to other diptera and some species of ticks. In the work on the cultivation of trypanosomes, efforts were made to express all the parameters in quantitative terms, an approach which has not been associated with such work in hitherto published reports.

Chapter 2

REVIEW OF THE LITERATURE

'Tissue culture' techniques are said to have started with the simple experiments of Roux (1885) who was able to maintain for a few days the medullary plate of a chick embryo in warm saline. This, however, was not 'tissue culture' as it became defined in the twentieth century. For the purpose of this thesis the use of the term 'tissue culture' includes all the aspects of in vitro cultivation of organs, tissues and cells as recommended by Federoff (1966, 1967). Ross Harrison (1907) is credited with the first demonstration of the capacity of tissue explants to retain some of their physiological characteristics and to grow in non living media. He showed that when cultured in a bacteria-free environment the explanted pieces of tissue from the medullary tube region of frog embryos survived for several weeks in clots of frog lymph; moreover, axons grew out from the explants. From that time, tissue culture techniques have developed rapidly, this development having been given strong impetus by the introduction of antibiotics which now play a major part in preventing bacterial contamination. Today, the use of tissue culture methods has contributed effectively to research in the fields of virology, cell biology and biochemistry (Paul, 1970).

According to most modern investigators tissue culture techniques can be placed in the following three major categories: a) The earliest primary tissue explants which, when maintained in suitable media, often lose their original organization giving rise to outgrowths of actively dividing although very often "dedifferentiated" cells. b) Primary mono- or polytypic cell monolayers and cloned or

uncloned cell lines. In these the cell cultures are handled by techniques similar to those developed for micro-organisms.
c) Organ cultures consisting of entire organs or parts thereof in which growth by cell multiplication is of relatively minor significance, but which maintain their original histological architecture as well as their physiological attributes.

ARTHROPOD TISSUE CULTURE

For nearly 50 years since the first recorded success with vertebrate "tissue culture", cultivation of insect tissues had proved a most difficult task. The reasons for the difficulties have been reviewed by many workers who have offered a variety of explanations for the fact that, even at present, insect tissue culture methods are in relative infancy (Day and Grace, 1959; Jones, 1962, 1966; Maramorosch, 1962; Schneider, 1967; Brooks and Kurrti, 1971 and Chao, 1973). Despite the statements that extensive knowledge of the physiology and biochemistry of vertebrates had played an important part in the development of suitable media for the cultivation of vertebrate tissues (Martignoni, 1960), the spectacular success in this field appears to have depended to a marked degree upon the wide use of embryo and tissue extracts. Such biological extracts derived from insects or even vertebrates were found, however, to be useless for the cultivation of insect tissues. An important obstacle in the development of methods for insect tissue culture was the interest of the early workers in the maintenance of tissues for short periods rather than in long-term cultivation involving growth by cell multiplication. A good example of this type of system was the maintenance of insect spermatocytes in simple mixtures of salts and insect blood in which

maturation division could be followed (Goldschmidt, 1915; Lewis, 1916; Lewis and Robertson, 1916; Takakuson, 1924; Chambers, 1925; Belar, 1929; Baumgartner and Payne, 1930; Stern, 1940; Ris, 1949). Although these workers reported limited differentiation of insect tissues in vitro, tissues maintained in this manner cannot be considered as true tissue cultures.

There are several reports by early and more recent investigators (Glazer, 1917; Collier, 1920; Lazarenko, 1925; Taylor, 1935; Arvy and Gabe, 1946; Millara, 1946) who claimed to have grown in vitro insect tissues other than spermatocytes. These results could not, however, be confirmed (Goodchild, 1954). The difficulty with the results of Schmidtmann (1925) and Frew (1928) has been that these workers, among others, tended to confuse cell migration with cell division, bleb formation with mitosis, and clumping with aggregation. In view of this it is difficult to evaluate either the methods or the culture media used.

Trager (1935), using a medium containing ingredients known to favour growth of vertebrate tissues, observed migration of cells from explants of silkworm ovarian tissues. This moderate success stimulated numerous workers to attempt cultivation of various insect tissues in media suitable for vertebrate tissues. The insect material grown in culture included mosquito nerve tissue (Pfeiffer, 1937, 1939, 1943; Carlson, 1946), salivary glands and gut (Gavrilov and Cowez, 1941), as well as imaginal discs (Trager, 1938; Gottschewski and Fischer, 1939). In all the above instances, success was limited.

The investigators interested in tissue culture have regarded the knowledge of the chemical composition of blood as of paramount importance in designing suitable growth media. Since in insects,

blood bathes the tissues directly, the insect physiologists believed that haemolymph contained substances essential for growth. Accordingly insect blood was incorporated in culture media. This blood, however, proved extremely toxic to tissues, owing to the presence of the polyphenol-polyphenoloxidase system which activated in spilt blood, produces a deposition of highly toxic brown-coloured quinone substances (Dawson and Tarpley, 1951; Mason, 1955). The activity of the enzyme can be inhibited by heating the blood (Bodine, Tahmisian and Hill, 1944), and this method of inhibition was employed with considerable success by Wyatt (1956) in her attempt to grow silkworm ovarian tissues. This worker had at her disposal also much more information on the composition of insect blood (Bialaszewicz and Landau, 1938; Tobias, 1948; Buck, 1953; Duchateau, Florkin and Leclerq, 1953; Wyatt, Loughead and Wyatt, 1956) and using all this knowledge, she achieved by far the most successful cultures of silkworm cells, which were maintained in a healthy state for extended periods.

Wyatt's (1956) medium subsequently was modified by Grace (1958a, b) and Vago and Chastang (1958a), who further improved the growth of insect cells in vitro. The preparation of Grace's medium was simplified by Jones and Cunningham (1960, 1961) and Cunningham (1961) by replacement of individual amino acids and members of the Vitamin-B complex with lactalbumin hydrolysate and yeast extract respectively. Since that time equally suitable media have been devised for cultivation of tissues from leafhoppers (Hirumi and Maramorosch, 1964a, b; Chiu and Black, 1967), grasshoppers (Carlson, 1961), cockroaches (Ting and Brooks, 1965; Landarau, 1968), drosophila (Echalier, Ohanessian and Brun, 1965), mosquitoes

(Schneider, 1968a, b, 1969) and ticks (Martin and Vidler, 1962; Rehacek, 1962; Varma and Wallers, 1965).

Thus, during the last 10 years suitable media have been developed for growing tissues from different arthropods. Some of this media were designed on the basis of the chemical composition of haemolymph from a given arthropod; others were prepared by combining components of media used to grow invertebrate and vertebrate cells.

Cell lines

According to Suitor (1966), one of the most important aims of arthropod tissue culture was the establishment of continuously growing cell lines. Indeed, in the catalogue of invertebrate cell lines published by Hink (1972), cell lines from 35 insect species are listed. Among them Diptera are represented by 14 species, Lepidoptera by 10, Homoptera by seven and Dictyoptera by four. The total number of cell lines from all species is 85, of which 78 were reported between 1967 and 1972, the period of significant advances in the field of insect cell cultures.

The development of insect cell lines was attributed to the availability of successful methods for dissociation of various tissues. Trypsin, the enzyme used routinely with vertebrate tissues, was found unsuitable for those of insects by most early workers, although St. Amand and Tipton (1954) reported some success using this method with grasshopper neuroblasts. Separation of undamaged integument cells from Peridroma margaratosa with a hydrolytic enzyme-rich extract from the hepatopancreas and crop of the garden snail, Helix aspersa, was accomplished by Martignoni, Zitcer and Wagner, 1958. Other investigators who employed trypsin could reduce cell damage either by limiting the digestion period to 5-10 min. (Rehacek,

1963) or by reducing the concentration of the enzyme to 0·1% or less (Aizawa and Vago, 1959; Hirumi and Maramorosch, 1964a, b). Undissociated small fragments resulting from such trypsin treatments could be dissociated further without causing damage by subjecting them to the action of hyaluronidase (Aizawa and Vago, 1959). Trypsin has been used also in combination with EDTA for the dissociation of Drosophila embryonic tissues (Lesseps, 1965).

The first insect cell line was established by Grace (1962) who, using trypsinized ovarian tissue from Antheraea eucalypti succeeded in a slow adaptation over a period of 10 months of the cells to the in vitro environment. These results stimulated much activity among workers who established large numbers of insect cell lines (Grace, 1966, 1967; Chiu and Black, 1967; Singh, 1967; Mitsuhashi, 1967; Ohanessian and Echalier, 1967; Peleg, 1968a, b, c; Schneider, 1969; Varma and Pudney, 1969).

The time required for adaptation of cells from various insects to grow in cultures varied with the donor species. Four cell lines from A.eucalypti took 9, 10 and 11 months to become established. Only 6 months were needed to establish a line of haemocytes from Chilo suppressalis (Mitsuhashi, 1967), while cell lines from Aedes aegypti and Aedes albopictus required only short periods of adaptation (Grace, 1966; Singh, 1967).

Established cell lines frequently consist of more than a single type of cell. For some studies mixed populations are underirable. In such investigations pure cell lines derived from clones are the material of choice. The capillary technique (Sanford, Earle and Likely, 1948) and the dilution methods developed for vertebrate cells, were used successfully for cloning those from

Aedes aegypti and Antheraea eucalypti (Suitor, Chang & Liu, 1966; Grace, 1966). The cloned cell lines are especially well suited for studying the responses to physical and chemical factors or for determining the nutritional requirements of various cell types. Since such cloned cells cannot be considered as normal, results obtained with them ought not to be applied indiscriminately to other clone-derived cultures or to cells from the original host.

Applications of insect tissue culture.

Tissue cultures of vertebrates have been used as a tool for studies of genetics, morphogenesis and differentiation and cell physiology. They have also been found most useful for investigations of a variety of viral, bacterial and protozoan parasites. In the light of the significant progress achieved with vertebrate tissue cultures, it is not surprising that numerous investigators have tried to cultivate arthropod tissues in vitro. The fundamental biological problems could be attacked if suitable systems were developed as many arthropods serve as vectors of viruses, rickettsiae and protozoa pathogenic to man, his domestic animals and his crops. Glazer (1917) and Trager (1935) were among the first parasitologists to be successful in the cultivation of etiological agents of arthropod-borne diseases.

Studies of genetics. The usefulness of cell culture techniques for genetical analysis and the earlier achievements in this field made possible with the aid of these methods were discussed in "Cytogenetics of Cells in Culture" (edited by Harris, 1964). Insect cells in culture are especially useful for cytogenetics, because they have fewer chromosomes than the vertebrate cells and also because in some species at least (i.e. Drosophila) many of the loci have

been well established (Barigozzi, 1971). The established Drosophila melanogaster cell lines developed by several workers (Horikawa and Fox, 1964; Echalier and Ohanessian, 1968, 1969, 1970; Echalier, Ohanessian and Brun, 1965) provided excellent material for studies of chromosome structure, of differentiation between eu- and heterochromatin and of DNA replication (Halfer, Trepolo, Barigozzi and Ficcaro, 1969) as well as of chromosomal aberrations (Dolfini and Gottardi, 1966). Genetic control of the growth of cells derived from different inbred Drosophila stocks was also demonstrated in cultures in vitro (Rezzonico and Gottardi, 1967). Various methods whereby Drosophila cell cultures could be employed for chromosomal studies were outlined by Barigozzi (1972), who pointed out the importance of these in vitro systems in such investigations.

Morphogenesis and differentiation. The problems of differentiation in in vitro cultures of cells and organs from various animals, including insects and the achievements in this field of research have been outlined in several recent reviews (Marks, 1970; Brooks and Kurtti, 1971; Demal and Leloup, 1972; Takami, 1972).

Among the earliest studies included in the present section was that of Goldschmidt (1915) on mitosis and gametogenesis in Hyalophora cecropia gametocytes maintained in haemolymph. The attempts of Frew (1928) to cultivate blow fly imaginal discs in haemolymph, however, failed because of inadequate aseptic techniques and unsuitable culture media. Using external structure alone as evidence, Fischer and Gottschewski (1939), claimed to have observed partial differentiation in explanted imaginal discs of the wing, leg and eye from Drosophila, and similar results were published on pupal testes of this species (Stern, 1940). However, Goodchild (1954) achieved very little success

with epidermis, heart and testes of Rhodnius prolixus and Blatta orientalis. On the other hand, partial in vitro differentiation of imaginal leg and eye buds from Calliphora erythrocephala and D.melanogaster, respectively was observed by Demal (1955, 1956). Only maintenance, but no mitosis or maturation of ova was reported by Beckel (1956) in adult ovaries of three Aedes species. Gottschewski (1960) however, saw growth and differentiation of D.melanogaster eye buds maintained in culture.

Important studies on the behaviour of in vitro cultures of D.melanogaster larval organs irradiated in vivo were carried out by Horikawa and Sugahara (1960). Working with normal organs of C.erythrocephala, Demal (1961) reported contractions of the aorta and a limited differentiation of pro-nymphal ovaries and testes. Subsequently, Lender and Duveau-Hagege (1962, 1963a, b, 1965) observed in vitro differentiation of gonads from fully grown Galleria mellonella larvae and nymphs and an even more advanced differentiation of ovaries from Tenebrio molitor was achieved in culture by Lender and Laverdure (1967, 1968) and Laverdure (1967, 1969). Long-term survival of different organs as well as development of whole embryos of Blaberus cranifer was reported by Larsen (1963), while various investigators observed in vitro differentiation of D.melanogaster eyes, antennae, brains and ovaries (Schneider, 1964, 1965, 1966; Hanley, Fuller and Stanley, 1967; Mandaron, 1970). In a comparative study which involved examinations of histological preparations of cultivated and of well defined stages of non-cultivated gonads from C.erythrocephala, several investigators were able to verify the occurrence of in vitro differentiation (Demal and Leloup, 1963; Leloup, 1964, 1969, 1970; Leloup and Demal, 1968). Cell multiplication and proliferation of

tracheoles from tracheal fragments of Locusta migratoria were seen by Martin and Nishiitsutsuji-Uwo (1967).

The effect of hormones upon differentiation was investigated by many workers. In a study concerned with wound healing processes, nerve regeneration, and formation of cuticle in Leucophaea maderae, it was shown that cuticle deposition in regenerating legs was influenced by moulting hormones and the age of the tissues (Marks, 1968, 1970; Marks and Reinecke, 1964, 1965; Marks, Reinecke and Leopold, 1968; Marks and Leopold, 1970). Spermatogenesis was observed in germinal cysts from diapausing Samia walkeri and Platysamia cecropia pupae cultivated in haemolymph containing the growth and differentiation hormone (Schmidt and Williams, 1949, 1953). The effect of certain gases, metabolic inhibitors and temperature upon insect spermatogenesis in vitro was also studied (Schneiderman, Ketchel and Williams, 1953). Using Drosophila, Kuroda and Yamaguchi, (1956) and Horikawa (1960) were able to demonstrate that differentiation of larval buds and antennae was controlled by endocrines from the cephalic complexes and that the process varied among different strains of the fruit fly. The degree of in vitro differentiation was used as a measure of the hormone level in the flies' body fluid. Larval epidermis of Schistocerca gregaria was found to differentiate only in tissues that were in a certain stage of development (Miciarelli, Sbrenna and Colombo, 1967). Through the use of histological techniques, secretory activity of the neurosecretory cells was demonstrated in isolated brains of Calliphora erythrocephala (Leloup and Gianfelici, 1966; Gianfelici, 1968a, b), of Aeschna cyanae (Schaller and Meunieur, 1967) and Manduca sexta (Leloup and Marks, 1973). Some differentiation in the presence of

synthetic hormones was noted in cultures of organs of various insects (Agui, Yagi and Fukaya, 1969a, b; Burdette, Hanley & Grosch, 1968; Judy, 1969; Laverdure, 1969; Mandaron, 1970; Marks and Leopold, 1970; Oberlander and Fulco, 1967; Oberlander, 1969a, b; Sengel and Mandaron, 1969; Williams and Kambysellis, 1969; Yagi, Kondo and Fukaya, 1969).

Hormone action at the cellular level can be demonstrated in insect cell cultures (Marks, Reinecke and Caldwell, 1967; Kroeger, 1968). Marks (1970); Vaughn, (1971) and Cohen and Gilbert (1972), have reviewed this subject. This action appears to be specific since Grace (1958a, 1959) observed little or no effect with extracts from cockroach prothoracic and blow fly ring glands upon silkworm cell cultures.

Cultivation of parasites in arthropod cell cultures. Perhaps the greatest interest in arthropod and more specifically insect tissue culture was stimulated by the spectacular success achieved through the use of this method in the field of virology. Indeed, growth of viruses in vitro proved feasible and brought with it the possibility of vaccine production under rigidly controlled conditions. Although as early as 1917, Glazer attempted to grow the polyhedrosis virus in tissue culture it was Trager (1935, 1938) who succeeded in cultivating this virus of silkworms in silkworm tissue culture as well as that of western equine encephalitis (WEE) virus in surviving mosquito tissues. Similar attempts at cultivation of arboviruses were not reported for about 20 years at which time Japanese B. encephalitis (JBE) virus was grown in mosquito tissue culture (Price, 1956). Under these conditions the virus was found to lose its infectivity temporarily. Only survival was reported for eastern

equine encephalitis (EEE) cultivated in midgut cells from A.aegypti larvae (Haines, 1959). More satisfactory results were achieved subsequently by growing EEE in cells of primary cultures prepared from minced mosquito larvae.

A superior system for studies of arboviruses was provided by the development of insect cell lines. The titres of JBE virus increased 10-100 times after 3 or 4 weeks in cultures of Grace's Antheraea eucalypti cell cultures. This cell line has been found subsequently to support a large number of arboviruses, including that of yellow fever (UF) (Converse and Nagle, 1967; Yunker and Cory, 1968; Yunker, 1971).

The establishment of mosquito cell lines (Grace, 1966; Singh, 1967; Peleg, 1968b; Varma and Pudney, 1969; Schneider, 1969) provided a system especially suitable for the cultivation of arboviruses. Of these lines, the one developed by Singh (1967) was used most widely, because no insect haemolymph was needed in the medium. The various viruses grown in insect cell lines have been listed and discussed by Yunker (1971) and Singh (1971).

Extensive investigations have been conducted to ascertain the suitability of various cell lines to support the growth of different viruses. Rehacek (1968a, b) found that Sinbis, Semliki, Berbera, EdgeHill and Kokoberra viruses failed to grow even 24 days after inoculation into cultures. On the other hand, Murray Valley encephalitis (MVE), Japanese encephalitis (JE), West Nile and Kunjin viruses multiplied in the cells causing cytopathological changes. Aedes albopictus and A.aegypti cell lines of Singh supported growth of different viruses some of which caused cytopathic effects (Singh and Paul, 1968a, b; Buckley, 1969; Paul, Singh and Bhat, 1969;

Yunker and Cory, 1969). High titres of EEE, Semliki Forest and West Nile viruses were reported from cell lines derived from A.aegypti by Peleg (1968b); However, no cytopathic effects were noted.

Development of techniques for growing tick cells (Rehacek, 1958, 1962, 1965a, b; Rehacek and Hana, 1961; Martin and Vidler, 1961) made studies of tick-borne viruses possible. The achievements with these viruses were summarized by Rehacek (1971, 1972).

Progress in the cultivation of arthropod-transmitted plant viruses in vector tissue culture has been made since aphid and leafhopper tissues were grown successfully as primary explants by several investigators (Mitsuhashi and Maramorosch, 1964; Mitsuhashi, 1965a; Chiu, Reddy and Black, 1966). Even more useful was the introduction of cell lines of various species of Homoptera (Hirumi, 1971; Hink, 1972).

Among the etiological agents of viral and related diseases of plants, that of asters yellows, found more recently to resemble mycoplasma-like organisms (Hirumi and Maramorosch, 1969) could not be recovered from tissue cultures of its natural vector, the leafhopper Macrosteles fascifrons (Maramorosch, Mitsuhashi, Streissle and Hirumi, 1965). Limited survival of the "virus" occurred, however, in the cells of another leafhopper Agallia constricta and more recently successful growth of the organism has been achieved in primary cultures of both species (Hirumi, 1971). The rice dwarf, would tumour and potato yellow dwarf viruses have been cultivated in embryonic and adult tissue cultures of their leafhopper vectors (Mitsuhashi and Nasu, 1967; Chiu, Liu, MacLeod and Black, 1970) but the need for the development of additional vector cell lines has been

emphasised by Hirumi (1971) and Mitsuhashi (1972).

After it was demonstrated that vertebrate cell cultures used widely for growing viruses were less suitable for the propagation of rickettsiae, the interest of many investigators was turned towards arthropods as the possible source of cells which would support in vitro growth of these latter parasites. Since ticks are frequent vectors of rickettsiae, their tissues were employed in a number of experiments. Thus Coxiella burneti was cultivated successfully in explanted organs and primary tissue cultures of Hyalomma asiati, H.inirmis and Dermacentor marginatus (Rehacek and Brezina, 1964; Kordova and Rehacek, 1965). Primary cultures of trypsin-dispersed H.dromedarii cells were found to be very susceptible to infection with Rickettsia conori, R.akari, R.prowazeki and R.mooseri; the tick cells were suitable also for studies of the cytopathogenic effect of the rickettsiae (Rehacek, Brezina and Majerska, 1968). Tick cell cultures support multiplication of the Rocky Mountain spotted fever rickettsia (Rehacek, Zupancicova, Brezina and Urvolgyi, 1973) and various pathogenic rickettsiae including R.tsutsugamushi and C.burneti can be cultivated with success in Grace's Atheraea and Singh's Aedes albopictus and some other cell lines (Yunker, Ormsbee, Cory and Peacock, 1970; Yunker, and Cory, 1973). That the cultivation of Rickettsiae in arthropod cell lines could be useful in the production of experimental rickettsial antigens and vaccines free from factors known to interfere with serological reactions was postulated by Yunker and Cory, (1973).

Of special significance in this dissertation are the previous results on cultivation of parasitic protozoa in the presence of insect tissue cultures. Trager (1959a, b) succeeded in cultivating

Trypanosoma vivax, T.congolense and T.brucei in the presence of Glossina palpalis tissues in vitro. By placing culture forms of T.vivax overnight at 37°C, he reported that he was able to produce trypomastigotes which on microscopical examination were found to resemble the metacyclic stages and which produced an infection in a sheep. No infection developed, however, when culture forms of T.brucei and T.congolense were handled in a similar manner and inoculated into laboratory rodents and a sheep respectively. Subsequently, Nicoli and Vattier (1964) grew Trypanosoma rhodesiense in tissue cultures of G.fuscipes. Their insect tissue cultures were less successful than those of Trager, despite the fact that they used his medium, and no infectivity to laboratory rodents was demonstrated by the midgut-like trypanosomes. The most recent investigations on Glossina morsitans (Cunningham, 1972, 1973a, b) resulted in the development of very satisfactory methods for the in vitro cultivation of various tissues and organs from this species of tsetse. Several salivarian and stercorarian species of trypanosomes grown in the presence of tsetse tissues produced rich populations consisting of organisms morphologically similar to some of the forms found in the alimentary tract of the vectors infected with the respective species.

No development of the Plasmodium oocyst was achieved in attempts at in vitro cultivation of oocyst-bearing mosquito gut (Ball, 1947 1948, 1954; Ragab, 1948, 1949). It was demonstrated later that development took place in Plasmodium relictum oocysts isolated completely from the alimentary tract (Ball and Chao, 1957). Since the mosquito gut is non-sterile, progress in cultivation of the malaria parasite in this system was not possible until the development of axenic methods for rearing mosquitoes had been achieved (Akov, 1962;

Boorman, 1967). Although development of sporozoites in Plasmodium gallinaceum oocysts was observed in modified Grace's medium in the absence of cells (Schneider, 1968a), the rate of development was improved by the presence of such cells (Schneider, 1968b). Very rapid development of P.relictum oocysts in mosquito cell lines also was reported (Ball and Chao, 1971). Yet, according to Ball (1972) primary cell cultures from mosquitoes might be more suitable than the more highly selected pure cell lines for development of the malarial parasites.

The first attempts to grow Nosema bombycis, the microsporidian causing a serious disease in silkworms, in cultures of tissues of this insect, resulted in failure. Successful results with this system were obtained, Ishihara and Sohi (1966) and Kurtti and Brooks (1971) who released the sporoplasms by treating the spores with dilute alkali (after Ohshima, 1937), before inoculation of the insect cell cultures.

At the present time the knowledge of haemolymph composition, the availability of commercially prepared media, and the other advances in culture methods have contributed to the recent successes in arthropod tissue culture techniques, now far more dependable and useful in studies of a variety of parasitic organisms.

THE CULTIVATION OF TRYPANOSOMES

The salivarian and stercorarian trypanosomes (Hoare, 1964) undergo important morphological and physiological changes in the vertebrate and invertebrate hosts. Yet, even today the factors controlling the morphogenetic and biochemical events are poorly understood. Studies of these events at the biochemical level

require large quantities of different stages, not available, particularly, in the invertebrate vectors. Development of different kinds of suitable culture methods would facilitate investigations of various aspects of morphogenesis.

Several reviews of cultivation of Trypanosomatidae have been published during the last decade (Jadin and Wery, 1963; Guttman and Wallace, 1964; Tobie, 1964; Bishop, 1967; Trager and Krassner, 1967; Taylor and Baker, 1968; Trager, 1968; Jadin and Le Ray, 1969; Zeledon, 1971; Fromentin, 1971). The aims of culture have been an aid to diagnosis, elucidation of nutrition and metabolism and studies of morphogenetic and biochemical events during transformations accompanying changes of habitat from vertebrate hosts to invertebrate vectors. In the present review, cultivation of species pathogenic to man and domestic animals will be emphasized.

The work "culture"has been used rather indiscriminately, and includes those instances in which the parasites merely survive and those in which they actually multiply. Weinman (1953) attempted to distinguish between the two types by using the terms "isolation medium" permitting the establishment of a trypanosome strain, and "maintenance medium" supporting the prolonged growth of the parasites. Since neither of these terms are very applicable, they have not been accepted in the literature. Actually "growth" and "maintenance medium" used commonly in tissue culture literature are preferable.

Development of culture media. At the beginning of this century MacNeal and Novy (1903, 1904) devised a medium in which Trypanosoma lewisi and T.brucei multiplied in the condensation water (water of syneresis) collecting at the base of nutrient blood agar

slopes. Shortly thereafter this medium was modified by Nicolle (1908) who omitted beef extract and peptone. He found that Leishmania tropica grew in the water of syneresis on the surface of a slope consisting of agar, marine salts and rabbit blood. This medium, known as the original NNN (Novy - MacNeal - Nicolle) has been used with considerable success for Leishmania, T.cruzi and several pathogenic salivarian trypanosomes. A further modification of NNN was Weinman's medium (1944, 1946), which contained human rather than rabbit blood. Another descendant of NNN medium was that of Tobie, von Brand & Mehlman (1950). This truly biphasic medium has been used in its original form or with slight modifications for the cultivation of Leishmania spp. as well as of salivarian and stercorarian members of the genus Trypanosoma.

Another line of development has involved completely liquid media. The mixtures designed by early workers were used successfully for Trypanosoma gambiense (von Razgha, 1929; Reichenow, 1934) and for this species as well as for T.brucei and T.rhodesiense (Brutseart and Henrard, 1938) included a balanced physiological salt solution and whole blood of several mammalian species, including man. These media had an important disadvantage in that the parasites tended to adhere to the red cells, resulting in a problem of harvesting pure trypanosome suspensions. Subsequently, Pittman (1970) and Dar (1971) developed liquid media containing lysates for T.rhodesiense and T.brucei. More recently Cross (1973) and Cross and Manning (1973) cultivated T.rhodesiense and T.brucei in a partially defined liquid medium.

Attempts to cultivate bloodstream trypanosomes.

Demarchi and Nicoli (1960) reported growth of T.gambiense and T.rhodesiense in the presence of several mammalian cell lines, and

similar attempts were made later by other workers (Fromentin, 1961; Le Page, 1967; Ward and West, 1970; Hawking, 1971; Mendez and Honigberg, 1972). In all instances the survival of the bloodstream trypomastigotes was extended somewhat over that observed in non-living media, and limited multiplication of such forms was reported by Le Page (1967) and Hawking (1971).

Of special significance to the present studies have been investigations in which salivarian species were cultivated in the presence of Glossina tissues (Trager, 1959a, b; Nicoli and Vattier, 1964; Cunningham, 1973 a, b; See the previous chapter of this thesis for details of these reports).

Cultivation in chick embryos. After Biocca (1938) succeeded in growing T.brucei in chick embryos, this method has been used successfully for the cultivation of several other species. (Langley, Clausen and Tatum, 1935; Hood, 1949; Goedbloed & Southgate, 1969; Dar, 1971). Recently T.brucei was reported to retain infectivity to mice after being maintained for 10 days in the embryos (Dar, 1971).

Infectivity of cultures. Although it is possible to cultivate many salivarian trypanosomes for extended periods in a variety of growth media, the flagellates, which morphologically resemble the forms found in Glossina midgut, are not infective to laboratory rodents. As has been suggested by some of the earlier workers (for example von Razgha, 1929), the infectivity of the cultures appears to be retained for only as long as bloodstream forms survive in a population (for review, see Mendez & Honigberg, 1972). The stercorarian species are in general much easier to cultivate in media used for the Salivaria, and some have been observed to complete their life cycle

including the development of metacyclic stages. The members of the Stercoraria which have been tested for infectivity in the course of cultivation, i.e. T.lewisi, T.musculi (T.duttoni) and T.cruzi, were found to retain their infectivity to laboratory rodents for prolonged periods. In NNN medium T.cruzi was reported to remain infective for 13 years (Packchanian & Sweets, 1947). When maintained in the presence of mammalian cell cultures, this latter species is found in flagellated stages free in the medium and also as amastigotes within the cells (for review, see Trager and Krassner, 1967). Other stercorarian species, i.e. T.melophagium and T.theileri, have also been maintained in vitro in the NNN or Tobie et al's (1950) medium (Herbert, 1965; Gray & Nixon, 1967; Wells, 1969); however infectivity of these cultures has not been ascertained experimentally. Among the species from birds and amphibians which have not been placed in either of the major sections by Hoare (1964), T.avium, T.mega and T.rotatorium are easily maintained in diphasic media, but their infectivity after in vitro growth has not been tested.

The application of culture methods to diagnosis

Since the stercorarian species grow well in vitro, cultivation methods have been found very useful in diagnosing infections with these parasites, for example T.theileri in cattle and T.melophagium in sheep (Herbert, 1965; Gray and Nixon, 1967; Wells, 1969) which occur in very small numbers in blood and are thus seen only rarely on microscopic examination.

Since the Salivaria are more difficult to cultivate, their growth in various media cannot be used as dependably as that of Stercoraria for diagnostic purposes. Yet T.gambiense was grown in

monophasic liquid media as early as 1938 by Brutsaert & Henrard and subsequently by Pinto (1952, 1953, 1954). An improved method for the isolation and cultivation of T.rhodesiense and T.gambiense was devised by Weinman (1960a). His medium consisted of nutrient human blood agar and contained also polyvinyl sulphuric acid as anticoagulant. The flagellates in various morphological forms grew in the water of condensation on the surface of the agar slope.

Cultivation as a tool in physiological and biochemical studies. It has been pointed out recently by Brown, Evans and Vickerman (1973) that the striking structural similarities between the trypanosomes found in the midgut of infected Glossina and the fully transformed culture trypomastigotes need not necessarily reflect similarities in the physiology of these forms. Moreover, the morphological and biochemical changes occurring in the course of transformation of the bloodstream to culture stages do not have to be the same as those that take place in the insect vector. Yet the tacit assumption has been made by most investigators that the culture and tsetse midgut forms are physiologically identical and that the same applies with regard to the structural and biochemical transformations. Indeed, all the biochemical studies of the "procylic" trypomastigotes as well as of the functional aspects of transformations of the bloodstream to culture forms have involved the in vitro systems only (for reviews of these studies, see Honigberg, 1967; Srivastava and Bowman, 1971; Evans and Brown, 1971, 1972a, b, 1973; Newton, Cross and Baker, 1973; Brown, Evans and Vickerman, 1973).

Among the stimuli for transformation of the bloodstream to culture forms of the salivarian and stercorarian trypanosomes, temperature change has been considered to be of major importance

(Deane and Deane, 1961; d'Alesandro, 1962, Deane and Kirchner, 1963; Ristic and Trager, 1958; Bishop, 1967; Trager and Krassner, 1967). However, it has been demonstrated recently, at least in one strain of T.rhodesiense, that such transformations followed by multiplication of the culture forms can take place at 37°C (Honigberg and Gabre, 1972). Transformation and subsequent growth occurred in those instances in which the growth medium was supplemented with chick embryo extract and glycerophosphate.

The gradual metabolic changes and the accompanying structural transformations of salivarian trypanosomes, especially in cultures, have been discussed in numerous review articles (Honigberg, 1967; Vickerman, 1971, 1972; Newton, Cross and Baker, 1973). The morphometric measurements using the light microscope have been reported most recently by Brown, Evans and Vickerman (1973), whose paper also deals with fine structural and biochemical data. Electron microscopic studies started by Vickerman (1962) have been extended by him (Vickerman, 1968, 1969, 1971, 1972) and other workers (Steiger, 1973). These investigations have dealt primarily with the development of the posterior mitochondrial tube and of cristae in the entire chondriome during the morphogenetic process occurring during the transformation from bloodstream to culture forms. They have also been concerned with the loss by the culture forms of the surface coat (Vickerman, 1969; 1971, 1972; Steiger, 1973). This coat has been said to contain the varient antigens (Vickerman and Luckins, 1969). The biochemical changes were found to involve a gradual switch from cyanide-insensitive to cyanide-sensitive metabolic pathways, with the concommitant appearance of functional enzymes of the tricarboxylic acid cycle

and of cytochromes (for reviews of older work see von Brand, 1951; Honigberg, 1967; for reviews of the more recent findings, see Srivastava and Bowman, 1971, 1972; Evans and Brown, 1971, 1972a, b; 1973; Brown, Evans and Vickerman, 1973). Differences in utilization of energy substrates, especially glucose versus proline, in the course of transformation also was investigated (Srivastava and Bowman, 1971, 1972; Evans and Brown, 1972b). The origin, nature and composition of the variant antigens which have been studied with the aid of immunological and immunochemical means by numerous workers (see Gray in Weitz, 1970; Vickerman, 1971, 1972 for reviews) still remain to be fully understood. However, it appears relatively certain that these antigens are located in the surface coat which invests the bloodstream and metacyclic forms.

Infectivity in in vitro systems.

Infectivity, which appears to be closely related to certain structural and antigenic properties of the bloodstream and metacyclic forms in Salivaria, has been reviewed in several recent papers (Vickerman, 1969, 1971, 1972; Mendez and Honigberg, 1973). The alleged role of certain carbohydrates and amino acids in stimulating acquisition of infectivity by culture forms (Weinman, 1957; Geigy and Kauffman, 1964) has been disproved (Bowman, von Brand and Tobie, 1960; Amrein, Geigy and Kauffman, 1965). The effect of blood from various donors (Amrein and Hanneman, 1969) also is difficult to explain in rational terms. Yet, despite the fact that most investigators have been unable to produce infectivity in cultures of salivarian trypanosomes (for review see Mendez and Honigberg, 1972), the sporadic appearance of infectivity in in vitro systems has been reported on occasion not only by early authors (Novy and MacNeal,

1904; Behrens, 1914), but also by some of the more recent workers (Amrein, Geigy and Kauffman, 1965; Amrein and Hanneman, 1969; Cross, 1973). Further studies are required to elucidate this problem.

Another approach to producing infectivity in vitro has involved the cultivation of salivarian species in the presence of a variety of vertebrate cells. Trypanosoma brucei (Le Page, 1967; Ward and West, 1970, Hawking, 1971), T.rhodesiense (Demarchi and Nicoli, 1960; Jadin and Pierraux, 1960; Mendez and Honigberg, 1972) and T.gambiense (Demarchi and Nicoli, 1960) maintained for various periods in laboratory animals or culture media since their isolation were used by different workers. Irrespective of the type of cell culture or of the strain, species or the past history of the isolate studied (i.e. fresh isolate, numerous passages in laboratory animals, prolonged cultivation in vitro) the experiments were not successful with regard to the extension of infectivity to laboratory rodents.

Among the workers who cultured the salivarian trypanosomes in the presence of Glossina tissues (Trager, 1959; Nicoli and Vattier, 1964) only Trager (1959) was able to produce infective stages. His success was limited, however, to T.vivax; no infectivity occurring in similar cultures of T.congolense and T.brucei. The details of these experiments are discussed elsewhere in this thesis.

In the stercorarian species the situation is quite different from that observed in Salivaria, infectivity persisting for prolonged periods in vitro (Packchanian and Sweets, 1947; Ristic and Trager, 1958; see also Honigberg, 1967 for review).

Chapter 3

MATERIALS AND METHODS

Arthropods and their maintenance

Diptera

Glossina spp. Newly deposited pupae of *G.morsitans morsitans* (Westwood), *G.morsitans submorsitans* (Newstead), *G.austeni* (Newstead) and *G.palpalis palpalis* (Robineau - Desvoidy) were used. Except for *G.palpalis* which came from Dr. K. Riordan, Nigerian Institute for Trypanosomiasis, Kaduna, all pupae were supplied by the Tsetse Research Laboratory, Langford, Bristol.

The development of *Glossina* spp. has been described by Glasgow (1970) and Saunders and Phelps (1970). Puparium formation is followed by larval-pupal and pupal-adult apolyses (Hinton, 1971), the latter giving rise to a pharate fly. Throughout this report however the ages of pupae refer to the ages of puparia and their contents at different times after larviposition.

In all instances the puparia were placed in pots of sterile sand and incubated at 25°C at a relative humidity ranging from 65 to 80%. Under these conditions flies emerged from the puparia in about 28 days.

Aedes aegypti (Linnaeus). Eggs laid on filter paper were supplied by Dr. Susan Beasley, Zoology Department, University of Edinburgh. They were used to rear the mosquitoes aseptically by Boorman's (1967) method modified as described below. This procedure was necessary owing to the difficulties caused by micro-organisms found in the alimentary tract of laboratory-reared mosquitoes.

The following method in which all glassware and solutions were sterile was used. The filter paper covered with eggs was cut up into

pieces approximately 1 inch square. They were surface-sterilized in White's solution (Trager, 1959) for 15 minutes, washed three times in distilled water and placed in a Universal bottle containing 15 ml. distilled water. The bottle with a loose fitting cap was transferred to a vacuum dessicator and subjected to negative pressure. After 30 minutes most of the larvae had emerged. They were transferred by a pipette into 8 oz. medical flat bottles each of which contained a nutrient medium consisting of the following solutions: one gm. of bread crumbs in 80 ml. distilled water sterilized at 10 lb. for 10 minutes and (2) 20 ml. of a 5% "Marmite" (yeast extract) solution sterilized by Millipore filtration. The larvae, incubated at 25°C were provided with fresh medium every 3 days. At pupation, the insects were transferred into petri dishes containing distilled water. The dishes were then placed in a Geigy cage which included also a pad of sterile gauze saturated with 5% glucose solution. The cage was put in a sandwich box previously sterilized by ultra violet irradiation and incubated at 25°C.

Sarcophaga argyrostoma (Robineau-Desvoidy 1830). Pupae were obtained from the Zoology Department, University of Edinburgh. They had been stored at 4°C for varying periods. On arrival at the C.T.V.M. the pupae were placed in a ventilated jar and incubated at 25°C, 60-80% relative humidity.

Acarina

Rhipicephalus spp. R.appendiculatus Neumann, 1901, originating from the East African Veterinary Research Organization, Muguga, Kenya and R.bursa (Canestrini and Fanzago) from Professor K.T. Freidhoff, Institut für Parasitologie, Hanover, Germany were used.

Amblyomma hebraeum (Koch). This species was supplied by the Wellcome Research Laboratories, Berkhamstead, Herts.

Ixodes ricinus (Linnaeus). This species was collected from cattle on the island of Raasay, Inverness-shire.

With the exception of I.ricinus, all the ticks were maintained by Dr. D. Branagan, C.T.V.M. by the method of Bailey (1960).

Preparation of arthropods for culture of their tissues

Diptera

The adult Diptera were immobilised at about 5°C for 30 minutes. Flies, puparia and ticks were then placed in a sieve and held under running cold tap water for 1 minute. They were surface-sterilized in 10% Roccal for 10-15 minutes. Since the insects tended to float in this solution, they were immersed by gentle agitation of the flask every 2 or 3 minutes. They were then washed three times in insect balanced salt solution (IBSS) (Jones and Cunningham, 1961) containing 200 I.U./ml. penicillin, 200 µg/ml. streptomycin and 5µg/ml. Fungizone. The insects were dissected in a petri dish containing about 2 ml. culture medium.

Acarina

After having been sterilized in 10% Roccal and washed three times in IBSS, the ticks were transferred to a petri dish containing a piece of sterile filter paper and allowed to dry. They were embedded, dorsal side uppermost, in a depression of melted black dissecting wax in a petri dish and covered with culture medium. The dorsal integument was removed by cutting round the periphery with a Swann Morton scalpel exposing the tissues to be cultured.

Preparation of insect extracts and haemolymph

Glossina pupal extract

Heated extract. After having been washed and sterilized, at least 20 G.morsitans pupae, 8-10 days-old were removed from their puparia and placed in a centrifuge tube. The pupal tissues were crushed gently with a Pasteur pipette and centrifuged at 10000 g. for 15 minutes at 5°C. The clear supernatant fluid was removed carefully with a pasteur pipette, to avoid the transfer of the surface lipid layer, and heated at 60°C for 5 minutes to inactivate the toxic polyphenoloxidase system. After inactivation, the extract was centrifuged as before and the supernatant fluid stored at -20°C.

Non-heated extract. Four or five surface-sterilized puparia aged 8-12 days were crushed in 1·0 ml DS medium (Cunningham, 1973). The mixture was centrifuged at 2000 g. for 10 minutes and the clear supernatant fluid was decanted and used as culture medium.

Silkworm haemolymph

Final instar larvae of Antheraea pernyi, supplied by Worldwide Butterflies Ltd., Compton, Dorset were surface sterilised with cotton wool saturated with absolute ethanol. An incision was made on one proleg and the haemolymph collected in a centrifuge tube immersed in ice. The inactivation of the polyphenoloxidase system and subsequent treatment of the haemolymph was the same as described above for pupal extract.

Culture media

Several culture media were used for the cultivation of arthropod tissues and organs and of the protozoa. Their composition and methods of preparation are described in the Appendix.

Sterilization

All glassware was soaked overnight in 0·5% Decon 75, rinsed in six changes in warm tap water and three changes of distilled water. Instruments and glassware were sterilized either by dry heat at 180°C for 1 hour or in an autoclave at 15 lb./sq. in. for 20 minutes. Some solutions required filtration and Millipore Sterifil filters with membranes of 0·22 μm pore size were used.

All manipulations were carried out in a Microflow laminar flow clean air work cabinet, the working surface of which was wiped with 10% Roccal before use.

Dissection of salivary glands and the complete alimentary tract of tsetse flies

After sterilization of their surfaces with 10% Roccal, pupae were placed in petri dishes containing either dissecting solution (DS) or IBSS. The puparia were then ruptured with watch maker's forceps, the pharate fly being thus released. By grasping its thorax with forceps a fly was held firmly with its ventral side upwards. The head was held with another pair of watch maker's forceps and pulled away from the thorax with a gentle but steady motion in a straight line. In most instances, the long, thread-like salivary glands remained attached to the head. Upon removal of the head, the thorax was separated carefully from the abdomen revealing the crop, proventriculus and the anterior part of the midgut. Then, the integument at either side of the anal opening was torn with the forceps and the alimentary tract, including the proventriculus and crop, was pulled through the abdomen wall. After removal of the adherant fat body, the complete alimentary tract was transferred to a drop of culture medium.

Preparation of arthropod tissue cultures

Hanging-drop cultures. The tissues to be cultured were placed in small drops of culture medium on 22 mm^2 coverslips. If many cultures were prepared at one time, the coverslips were kept in closed petri dishes to prevent evaporation of the medium. Small spots of vaseline were placed between the wells in order to secure the position of the coverslips over the cavities of a Butt slide (Fig. 1). The coverslips were inverted over the cavities and sealed with a 1:3 mixture of melted vaseline and paraffin wax. Finally the slides were inverted and the cultures were incubated as "sitting-drops".

Leighton tube cultures. The material to be cultured was placed in a small drop of culture medium spread as a thin film over the surface of coverslips (9 x 35 mm) which were then placed in Leighton tubes and the caps were firmly screwed on.

Cultures of cells from trypsinized tissues. Engorged nymphs with conspicuous adult epidermis and appendages at the anterior end (5-8 days after immobilisation) (Fig. 2), were embedded in wax as described previously. The dorsal integument was removed and the alimentary tract, Malphigian tubules and rectum discarded. Tissues comprising mainly epidermis, appendages and the central ganglion were removed from the ventral part of the body (Fig. 3), washed in sterile IBSS and placed in about 0·5 ml IBSS. They were then cut into small fragments which were subjected to digestion by trypsin (0·25% Difco 1:250 in IBSS prewarmed to 37°C) for 3 minutes. After this period, the fragments were broken up by gentle agitation with a Pasteur pipette until the suspension became fairly cloudy. To arrest the trypsin action foetal bovine serum was added to about 20% final concentration. The suspension was then centrifuged at 80 r.p.m.

(150 g) for 5 minutes. The supernatant fluid was discarded and the pellet of cells suspended in culture medium and washed twice by centrifugation. The washed cells were resuspended in fresh medium and their concentration adjusted to about 10^6/ml. Cells from 50 ticks provided material for inoculation of 1 ml of suspension into each of three Leighton tubes or plastic test tubes. Incubation was at 28°C.

The primary cultures could be subcultured twice. In each instance, the following procedure was followed:

1) the medium was removed and the attached cells washed once with IBSS. Trypsin solution (as described above) was added to the cells for 10 minutes, after which the tubes were shaken vigorously, but without frothing the solution, to detach the cells from the substrate. Any remaining attached cells were scraped off with a rubber "policeman". The resulting suspension was centrifuged at 1200 r.p.m. (200 g) for 5 minutes and the supernatant fluid discarded. The cell pellet was resuspended in fresh culture medium and the cell concentration adjusted to 10^6/ml. One ml of the suspension was dispensed into each tube.

After the cells were attached to the walls of the culture tubes or to coverslips in about 5 or 6 hours, their growth was estimated with a Chalkley point graticule placed in a 10x eyepiece. Using this eyepiece and a 10x objective on an inverted microscope six random fields were selected and in each the number of dots superimposed upon cells was counted. The counts were repeated every 2 days. As the cells multiplied and spread over the substrate, the number of dots superimposed upon cells increased. This method of estimating

cell growth was particularly useful in studies in which only relatively small numbers of cultures could be prepared at one time. It obviated the need for discarding cultures after an individual count, as is necessary in the commonly used methods involving electronic counters, haemocytometers or packed cell volumes.

Cultivation of trypanosomes

In Butt slides. The cultures were either inoculated with stabilates or with blood obtained aseptically from the tail of infected mice when the parasitaemia was high. The blood was diluted with culture medium to give a concentration of about 1000 trypanosomes/mm^3. A drop of between 15 and 20 μl of the suspension of trypanosomes was placed on 22 mm^2 coverslips and one piece of tissue from freshly dissected pupae or adult tsetse flies was added to each drop. Hanging drop cultures were prepared as described above. Suspensions of trypanosomes incubated under the above described conditions but in the absence of insect tissues served as controls.

In tissue culture chambers. Slide tissue culture chambers (Sterilin Ltd., Richmond, Surrey) (Fig. 4) were used to observe the growth of trypanosomes separated from tsetse pupal alimentary tract by a semipermeable membrane. Bloodstream forms were mixed with culture medium and a drop of about 15 μl of trypanosome suspension was added to the deeper well of the chamber (a). A silicone rubber O-ring for a Millipore Swinnex 13 filter (d) was used to support the sterile disc of visking dialysis tubing (c) which was placed over the trypanosome suspension. One complete alimentary tract (b) from a pupa older than 23 days was placed carefully on the dialysis membrane above the drop containing the trypanosomes. The tsetse tissue was

kept moist in a drop of 5 µl of the culture medium. The rim of the well was smeared with Vaseline and the chamber sealed with a coverslip (e). The perfusion channels were sealed with molten wax and the culture chamber placed in a petri dish containing cotton wool saturated with sterile distilled water to maintain humidity during incubation. In the course of microscopic observations water condensation which appeared on the coverslip was removed with the tip of a hot needle.

Mammalian hosts

Female white Swiss C.D.T.O. mice were obtained from A. Tuck and Sons Ltd., The Mousery, Rayleigh, Essex. Rats were supplied by the Small Animal Breeding Laboratory, University of Edinburgh.

Strains of trypanosomes

Trypanosoma (Duttonella) vivax

TREU 1101. This strain, isolated originally in West Africa, was supplied by Dr. J.M. Clarkson, Liverpool School of Tropical Medicine, as stabilates of blood from a calf infected with Glossina morsitans transmitted trypanosomes.

TREU 65. A derivative of the Desowitz and Watson (1951) rat-adapted strain. The origin of this syringe passaged strain is obscure.

Trypanosoma (Nannomonas) congolense

TREU 623 - Eve 4. Isolated from a bovine host during the East African Veterinary Expedition, 1968. The infected bovine blood was inoculated into mice.

TREU 694. Stabilates of infected mouse blood prepared 22 days after the infected fly fed on the mouse.

TREU 261. This strain, syringe passaged innumerable times in laboratory rodents, was obtained from Dr. R.W.F. Le Page, Cambridge in 1966. Its history is obscure.

Trypanosoma (Trypanozoon) brucei

TREU 667. This pleomorphic strain, with low virulence to mice was isolated originally in Kenya and subsequently passaged once in a rat. Blood obtained from the rat at first peak parasitaemia has been cryopreserved.

Etat 5. A cloned population, very virulent to mice, resulting from an infection of a mouse with a single organism of strain TREU 289. The history of the latter strain is given in McNeillage, Herbert and Lumsden (1969).

Trypanosoma (Trypanozoon) rhodesiense

TREU 788. This cryopreserved strain was derived from a mouse passage of EATRO 181H strain isolated originally from a man and passaged in rodents.

Trypanosoma (Trypanozoon) gambiense

Isolate L_2. From a man at Bida, Nigeria, by inoculating lymph-node biopsies into rats. The parasites were sub-passaged in adult rats and then adapted to baby rats. Trypanosomes adapted to baby rats were then inoculated into baby mice, the pooled blood of which was preserved as stabilates in liquid nitrogen.

Isolate R_3. From a man in Abade, Nigeria and adapted to rodents by the method described above for Isolate L_2.

Trypanosoma (Trypanozoon) evansi

TREU 381. This strain, isolated in 1969 from a horse in Colombia,

was inoculated into mice. Thereafter it was transmitted again to a horse and once more into mice. Stabilates were prepared from mouse blood.

Trypanosoma (Herpetosoma) lewisi

TREU 726. A strain prepared from blood of a rat infected with TREU 14 from the London School of Hygiene and Tropical Medicine in 1963.

Trypanosoma (Herpetosoma) musculi

TREU 1094. Isolated from Mus musculus brevirostris Waterhouse in Sicily in 1962 and syringe passaged many times in laboratory mice, was supplied by the Department of Medical Parasitology, London School of Hygiene and Tropical Medicine.

Trypanosoma (Megatrypanum) theileri

TREU 641. Originally isolated from an Ayrshire cow on blood agar cultures and subcultured many times was cryopreserved as TREU 439. A single organism of TREU 439 was inoculated into cultures and after 87 days the resultant population of trypanosomes was laid down as stabilates designated TREU 641.

TREU 644. Derived from the same Ayrshire cow as TREU 641 and subcultured on blood agar for 104 days before being preserved as stabilates TREU 443. A single organism from a culture of this stabilate was cultured and the resulting population preserved as TREU 644.

Trypanosoma (Megatrypanum) melophagium

TREU 89. This strain isolated from sheep keds by Dr. I.V. Herbert, North Wales, in 1960, has been maintained in blood agar

cultures or on sheep leucocyte cultures for an unknown number of passages. Stabilates of blood-agar cultures were used.

Histological methods

Demonstration of mitoses in tick tissues. Engorged nymphs R.appendiculatus of various ages were dissected as described in the section "Materials and Methods". The developing adult epidermis, mouthparts and appendages were removed and cut into small fragments in 2 ml of 0·075 M KCl. solution. After 4 minutes, the fragments were transferred to a centrifuge tube and broken up by mixing with a fine bore pasteur pipette until a cloudy cell suspension was obtained. The cell suspension was centrifuged at 1500 r.p.m. for 3 minutes, the clear supernatant fluid discarded and the cell pellet shaken. Fixative (3 pt. methanol: 1 pt. glacial acetic acid) was added slowly and left for 2 minutes. The fixative was changed 3 times, the exposure being 1 minute in each case. Ultimately the cells were resuspended in 0·2 ml and dispersed by gentle agitation with a pasteur pipette. One drop of the resulting suspension was placed on a slide pre-cooled in the freezing compartment of a refrigerator. The drop immediately spread to the edge of the slide and was left to dry at room temperature for 2 hours. Occasionally, the slides were dried over a spirit lamp flame. The application of heat tended to break up the cells and thereby liberate the chromosomes.

The preparations were stained in 1% lactic acetic orcein for 2 hours at 37°C, dehydrated by washing in cellosolve (2-ethoxy-ethanol), cleared in Euparal essence for 1 minute and mounted in Euparal.

Staining of tsetse and tick cell cultures. To retain the explants and cellular outgrowths on the coverslips care was exercised during washing and fixation. Before fixation the cultures were washed as follows. The culture medium was withdrawn with a fine pasteur pipette and replaced with a drop of IBSS to remove excess protein. This solution was then removed and the appropriate fixative added.

May-Grunwald-Giemsa. The preparations were fixed by adding a drop of absolute methanol. Thereafter the coverslips were tilted slightly to allow excess fixative to flow gently to one side where it was removed with a pipette. The cultures were stained with May-Grunwald solution for 10 minutes. After having been washed in distilled water the preparations were covered for 15 minutes with Giemsa's strain (Giemsa improved "R66") diluted 1:10 in distilled water. Washing in distilled water was followed by dehydration in 2 changes of acetone (2 minutes each) and partial clearing in a 1:1 acetone-xylene mixture (2 minutes). Clearing was completed in xylene (2 minutes) and the preparations were mounted in neutral canada balsam.

Oil Red O. The explants were fixed in 10% neutral formol saline and after washing in distilled water, stained with Oil Red O for 10-15 minutes in a closed petri dish. In all instances, the stain was freshly prepared by adding 4 volumes distilled water to 6 volumes of stain and allowing the mixture to stand for 5-10 minutes before use. It was then filtered directly onto the material to be stained. The stain was washed off with distilled water and differentiated in 60% ethanol for 1 minute. After a rapid rinse in distilled water the nuclei were counterstained in Cole's

haematoxylin for 3 minutes and "blued" in Scott's tap water.

Preparation and staining of tick organs. Organs obtained directly from ticks and those from cultures were fixed in Bouin's fluid for 12-24 hours. After washing in distilled water and several changes of 70% alcohol to remove the excess picric acid, the tissues were dehydrated in graded series of ethanol, cleared in xylol and embedded in paraffin wax. Then 7 μ sections were cut on a rotary microtome, the paraffin was removed with xylene and the sections were passed through a descending series of ethanol and stained with de-Groat's Haematoxylen for 5 minutes. Thereafter they were "blued" in Scott's tap water for 5 minutes and placed in 1% aqueous eosin Y for 30 minutes. The counterstained sections were washed in distilled water and transferred to a 5% solution of phosphotungstic acid for 30 minutes. A rinse in distilled water was followed by staining in 1% aqueous light green for 1 minute. After removal of the excess stain with distilled water, the sections were dehydrated in a graded series of ethanol, cleared in xylene and mounted in Canada balsam.

Staining of trypanosomes

Bloodstream forms. Thin blood films were dried and fixed in absolute methanol for 1 minute. They were placed for 40 minutes in Giemsa's stain diluted 1:10 in distilled water buffered to pH 7·2. The stained preparations were washed in distilled water and dried in air.

Culture forms. The coverslips were removed from the Butt slides and the insect tissues discarded. Small samples of culture medium containing trypanosomes were spread on slides and dried in air. The remainder of the medium was removed from the coverslips

which were then rinsed in IBSS and also dried in air. Both types of preparations were stained in Giemsa's stain by the method described for the blood stream forms except that the stain was diluted 1:20. The air dried coverslip preparations were ultimately mounted in green Euparal or neutral Canada balsam.

Measurement of growth of trypanosomes

To measure the growth of the trypanosomes, cultures were prepared in replicate. For each experiment, two or three cultures were examined and discarded daily. The medium was withdrawn into a capillary pipette and the trypanosomes washed off the coverslip with 9 volumes of IBSS. After thorough mixing a sample was introduced into a Neubauer haemocytometer and the number of trypanosomes per mm^3 counted. Each experiment was repeated on two or three occasions. The graphs represent the arithmetic mean and standard deviation of four or six counts. Only motile trypanosomes were counted.

Collection of Glossina haemolymph for amino acid analysis

Haemolymph was collected from three developmental stages of Glossina morsitans: 10-day pupae, pre-emerged flies and unfed flies 2 days after emergence. In all instances precooling of the pupae or flies at 4°C for 30 minutes combined with the addition of a few crystals of phenylthiourea to the collected haemolymph prevented melanization. Since slightly different methods were employed in collecting the haemolymph from the three stages, these will be described separately.

Pupal haemolymph. Ten-day-old pupae were washed in distilled

water to remove sand grains adhering to their surfaces. After drying, the pupae were cooled at 4°C. With watchmaker's forceps a small fracture was made at the anterior end of the puparium and a capillary tube inserted into the haemocoel through the reupture. Haemolymph entered the tube by capillary action and care was taken not to collect the gut. The contents of the tube were expelled onto crystals of phenylthiourea in a small petri dish held over an ice bath. Haemolymph from 10 pupae was pooled and centrifuged in haematocrit tubes at 12000 g for 10 minutes. The clear supernatant fluid was stored in a small tube at -20°C.

Haemolymph from Pre-emerged flies. Twenty-seven-day-old pupae were washed and precooled as were the younger pupae (see above). The anterior end of the puparium was removed with watchmaker's forceps. The flies everted the frontal sac, which, when fully extended was cut with iridectomy scissors. The haemolymph was collected in haematocrit capillary tubes and treated with phenylthiourea as above. Upon removal of haemocytes by centrifugation at 2000 g for 5 minutes, the haemolymph was stored at -20°C.

Haemolymph from unfed adult flies. The flies were immobilized by being kept at 4°C for 30 minutes. Haemolymph was collected into a capillary tube from the stump end of the femur of an amputated posterior leg. By applying gentle pressure to the thorax, the fly expelled about 2 µl of haemolymph. The haemolymph from 15-20 insects was treated with phenylthiourea, centrifuged at 2000 g for 5 minutes to remove haemocytes and stored at -20°C.

Amino acid analysis

Immediately before analysis, pooled samples of 10-20 µl of haemolymph were placed in small tubes and the volume adjusted to 2·5 ml with a solution containing 0·1 N HCl, 10% sucrose and 0·25% µ mole norleucine. One ml samples of this solution were analysed for free amino acid content using a Technicon NC-1 Amino Acid Analyser. The analyses were carried out by Dr. John S. Slater, Morcdun Research Institute, 408 Gilmerton Road, Edinburgh, 17.

Chapter 4

EXPERIMENTAL RESULTS

The cultivation of organs, tissues and cells of Glossina morsitans in vitro.

In suitable media primary vertebrate and invertebrate cell cultures can be obtained most easily from embryonic or developing tissues composed of undifferentiated cells and healthy organ cultures are obtainable from virtually any developmental stage of the donor (Paul, 1970; Vago, 1971, 1972a).

Until the report of Cunningham, 1971, tissues and organs from only one tsetse fly species, Glossina palpalis had been cultivated successfully by Trager (1959) in a complex medium designed specially for this purpose. This medium, however, was found less suitable for the maintenance of tissues from Glossina fuscipes (Nicoli and Vattier, 1964). Since no information is available on the cultivation of tissues of G.morsitans, studies were undertaken to test a variety of media for their ability to support primary cell and organ cultures of this species.

Methods

Cell cultures. Six 10-day old pupae were dissected in culture medium and brain, midgut, imaginal body wall, proventriculus and imaginal limb tissues removed. All organs were cut into fragments of approximately 1 mm^3 in size with fine tungsten needles. Each tissue fragment was placed in a small drop of culture medium spread as a thin film over a coverslip and left for 10 minutes to settle. Hanging-drop cultures were prepared.

Organ cultures. Pupae older than 18 days and newly emerged flies served as sources of alimentary tract (from the

crop to the rectum), head with attached salivary glands and reproductive organs. The adult flies were immobilised in the refrigerator (5°C) for 20 minutes. The organs were dissected and placed in hanging-drop cultures.

The tissues from six pupae were cultured at one time and three replicate experiments were performed with each of the culture media listed in Table 1. All cultures were inverted and incubated at 28°C as "sitting-drops" and the medium was changed every six or seven days.

Results

Cell cultures. Cell migration from explants occurred in six of the media tested (Table 2). Cellular outgrowths were noted when Jones and Cunningham (1960, 1961) medium was enriched with foetal bovine serum and tsetse pupal extract (media 2 and 3)[1]. Cell migration occurred also in cultures maintained in modified Trager's (1959) medium supplemented with these additives (media 8-10). Medium 9 supported outgrowths of cells in all the midgut explants and in about one-half of those of brain, imaginal body wall, proventriculus and imaginal limb tissue. Cell migration was observed in fewer explants cultured in Trager's medium supplemented with sheep serum (medium 10) or silkworm haemolymph (medium 11). No growth was noted however in media based on VP (Varma and Pudney, personal communication) (media 12 and 13), B.M.E. (Eagle, 1955) (media 14-16) except in medium 15 in which four out of 18 midgut cultures contained cellular outgrowths.

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1. In all instances, the numbers correspond to media and their modifications as listed in tables 1-3.

The best results were obtained using medium 9 and therefore the following descriptions are based on observations of cellular outgrowths from explants maintained in this medium.

Brain. Extensive cellular outgrowths were observed after about 4 days from 6-day pupae. The network of cells increased in area until the 9th day when no further outgrowth occurred (Fig. 5). The lissamine green viability test (Goldacre and Sylven, 1959) indicated that the elongate cells with delicate cytoplasm containing fine refractile granules remained healthy for 15-20 days when the medium was changed twice. Large, round fat cells were often present in the outgrowths, which were difficult to stain in situ since most of these explants containing such cells floated off the coverslips when washed in physiological solution before fixation.

Proventriculus. This part of the alimentary tract was conspicuous but not fully developed in 6-7 day old pupae. Proventricular explants frequently gave rise to outgrowths of fibroblast-like cells. Migration began on about the 3rd day of culture and the cells had spread a considerable distance from the fragments of tissue by the 6th day (Fig. 6). Mitoses were evident in stained preparations of cultures up to 12 days old (Fig. 7), but cell degeneration began after 14 days. Occasionally the centre of the tissue explants became motile and rhythmic contractions continued for 3 weeks.

Imaginal body wall. Presumptive imaginal body wall of 7-9-day old pupae produced outgrowths consisting of long narrow cells with central nuclei. Such cells appeared at the edge of

the explant after 3 days in culture (Fig. 8). They remained healthy for 12 days as indicated by the lissamine green viability test. Mitoses was not observed in stained cultures.

Imaginal limb-bud. The following cell types were observed in the cultures.

1. Fibroblast-like cells, which were observed initially on the 2nd day and had migrated some distance from the explants by the 6th (Fig. 9).

2. Small amoeboid cells, about 7 μm in diameter were seen at some distance from the explants (Fig. 10). These cells were often associated with large fat cells (Fig. 10).

Malpighian tubules. Cellular outgrowths from explants were seldom observed in cultures of tissues from 7-10 day old pupae. On one occasion on the 3rd day cells had spread out from an explant from a 10-day pupa (Fig. 11). The cells began to degenerate on the 8th day despite the addition of fresh medium on the 5th day.

Midgut. The anterior segment of the midgut of 6-10 day pupae contained a green fluid which was expelled before the tissue was placed in culture. Fragments of this tissue from 8-10 day pupae exhibited a different type of behaviour. After 2 or 3 days, large hollow vesicles were extruded from the edge of the explants (Fig. 11). These vesicles continued to increase in size until they had dwarfed the original explant. In dark phase contrast the nuclei of the cells were seen to be surrounded by dense clusters of droplets (Fig. 12). Staining with Oil Red O or Fat Red B indicated their lipid nature (Fig. 13). After 10 days, the vesicles began to contract rhythmically and these contractions persisted for up to 21 days when the culture medium

was changed three times.

Organ cultures. Viability of alimentary tract cultures was measured by active peristalsis of part or all of an organ. Healthy salivary glands displayed vigorous rhythmic contractions.

Alimentary tract. In the 16 culture media tested most of the cultures of alimentary tract of pupae older than 18 days exhibited peristalsis for 2 days. Media enriched with tsetse pupal extract supported peristalsis of the gut in a greater number of cultures and for longer periods of time than similar media without pupal extract (Table 3). Three out of 18 alimentary tract cultures in modified Trager's medium (medium 9) continued to contract for 30 days. There was a sharp decline in the viability of the tissues in these cultures on the 16th day. In the remaining media, many of the cultures had begun to deteriorate by the 4th day. In one of the cultures in medium 9 the crop duct had attached to the coverslip and cells began to "bubble out" from the edge of the duct on the 5th day. By the 7th day, elongate fibroblast-like cells had migrated from the tissue (Fig. 14). The cells remained viable for 10 days but were later dislodged from the coverslip by the active contractions of the crop.

Head with attached salivary glands and mouth parts. Table 4 indicates that cultures remained viable for the first 24 hours in most of the media tested. Peristalsis of the glands was noted, however, in only a few of the cultures in medium 12. In media supplemented with unheated tsetse pupal extract contraction of the glands was evident in some of the cultures for up to 3 days

and in medium 9 the glands remained alive for 7 days. A change of medium on the 5th day did not prolong viability of the cultures.

Testes. During development of the pharate fly within the puparium, the testes undergo elongation at about the 5th day, and by the 7th day they are coiled, each about 2 mm long. Cultures of testes of this stage of development did not exhibit peristalsis or cell migration in media listed in Table 1. Cultures of testes from pupae older than 23 days showed development of spermatozoa after 7 days in medium 9. Innumerable spermatozoa were released from the testes (Fig. 15) and they remained motile for up to 10 days. Similar cultures of testes from pre-emerged flies (26-27 day pupae) contained active free spermatozoa after 3 days and they remained viable for a further 7 days.

Ovaries. Cultures of ovaries from pupae at different stages of development did not show cell migration or peristalsis in the different media tested.

Chapter 5

Cultivation of Organs, Tissues and Cells of Ticks

The cultivation of tick tissues had received very little attention until Rehacek (1958) reported some success in growing cells from tissues of the engorged nymphs of Dermacentor marginatus. To study the cyclical development of Theileria parva within the tick vector, Martin and Vidler (1962) devised a successful method for the cultivation of tissues from Rhipicephalus appendiculatus, the cells which migrated from various tissue explants surviving for over 100 days. These workers, however, did not attempt to define the stages of development of the engorged nymphs which gave rise to the best cell cultures.

It was the aim of the present studies to ascertain which stage of development of the engorged nymph (= developing adult) was most suitable to yield prolific cell, and healthy organ cultures. It was assumed that tissues containing actively dividing cells would provide the most suitable material for culture, whereas more differentiated tissues from older engorged nymphs or developing adults would be less suitable. To test this hypothesis, actively developing tissues such as epidermis, appendages and central ganglion of engorged R.appendiculatus nymphs at various stages of development were examined for mitoses. Newly engorged nymphs were stored at 25°C and 80% relative humidity and were dissected at different times after immobilisation*, a stage

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* The immobilisation of engorged larvae and nymphs is regarded as being that stage after engorgement when the tick has lost the ability to make co-ordinated progress away from strong light as defined for R.appendiculatus by Branagan (1973).

at which the new adult epidermis and appendages begin to form.

The results of this test are presented in Table 5. Developing epidermis from engorged nymphs 2 to 7 days after immobilisation (I + 2 to I + 7) contained dividing cells which could be seen clearly in squash preparations stained with aceto-orcein (Figs. 16 and 17). Very little developing adult epidermal tissue was seen at stage I + 2. With increasing age this tissue became more evident until by I + 7 it had formed a veil-like cover over the entire body. Starting with stage I + 8, the dorsal epidermis was a conspicuous white sheet of tissue which did not contain mitotic figures.

Primary explant cultures

Effect of age of developing adults on production of cell and organ cultures. To study this relationship developing adults of various ages were used. The tissue explants consisted of the entire contents enclosed within the nymphal cuticle. The cultures were prepared according to the method detailed in "Materials and Methods", using VP_4 medium supplemented with 10% foetal bovine serum and containing penicillin and streptomycin. Tissues from two ticks were placed on a single coverslip, and whenever possible (see Table 6) 10 tubes were set up for each of the developmental stages to be tested. The cultures were incubated at 28°C and the medium changed every 7 days for a total period of one month, at the end of which the preparations were stained and examined for the presence of mitosis.

The result of the experiment is summarized in Table 6. As indicated in the table, only 12 to 17 out of 20 could be prepared successfully from the earlier developmental stages. In these

stages (I + 2 to I + 4) the gut tissue was very fragile and its rupture during dissection resulted in the liberation of gut contents which spread over the coverslips and prevented migration of cells. All such damaged tissues were discarded. Older stages were far more amenable to dissection and starting with I + 5, 20 out of 20 explants were successful.

The following three parameters were used in estimating the success of cultivation: cell migration, peristalsis of the alimentary tract and the ability of the cells to form monolayers over the entire surface of a coverslip. The cultures were examined every 7 days and the results are shown in Table 6 and summarized below:

Peristalsis (Table 6). In healthy cultures peristalsis of the gut diverticulum and rectum as well as the movement of the contents within the Malpighian tubules began after 3 to 7 days and increased in intensity by day 7, lasting up to the end of the experiment (28 days). In explants of the earliest developmental stages tested (I + 2 series) only 2 of the 12 cultures showed contractions. Among the remaining cultures of this series, none of which exhibited peristalsis, some had the gut contents spread over the coverslips, this spreading having occurred after the cultures were prepared. This condition might have been responsible for the lack of contractions. Peristalsis was observed in many more cultures resulting from older explants, I + 3 to I + 9, but in only 50% of those in the I + 11 series.

Cell migration and monolayers. It is evident from the results summarized in Table 6 that in terms of these two para-

meters the most successful cultures were obtained from explants of the stages I + 5. Of the earlier stages, I + 3 and I + 4 were more suitable for the establishment of successful cultures than I + 2. I + 7 produced cell monolayers only after 28 days, and no monolayers were noted in explants from stages I + 9 and I + 11.

In successful cultures obtained from stages I + 2 to I + 5 the cells began to migrate and form a small halo around the explants in about 3 or 4 days and within two weeks covered the coverslips forming monolayers. After 3 weeks the actively growing cells tended to pile up to form large aggregates distributed all over the surface of the coverslip (Fig. 18). In all cases in which monolayers were established the cultures could be maintained up to 106 days. Even in such relatively old cultures numerous mitotic figures were seen (Figs. 19, 20 and 21).

In all cell cultures it is most difficult to identify the cell types present in relation to those in the explant from which they have originated, because cultured cells tend to be morphologically very different from the tissue cells from which they are derived. In tick cultures many different cell types were seen (Fig. 22), fibroblast-like cells of different sizes being predominant. Many of them appeared to be actively phagocytic. They contained large numbers of eosinophilic granules (Fig. 23). Larger epithelial cells, presumably derived from the tick gut contained green-stained globules which probably represented products of intra-cellular digestion of blood (Figs. 23 and 24).

In some cultures sheets of epithelial cells grew out from the edge of the developing epidermis (Fig. 25).

Comparison of cultures grown in different media

Cell cultures derived from I + 4 and I + 5 developmental stages grown in VP_4 medium supplemented with foetal bovine serum (see the immediately preceding section) were compared with those grown in Martin and Vidler's B (1962) and in Eagles - Vago-Chastang (E-V-C) (Rehacek, 1965b). Although more difficult to prepare, the results in E-V-C medium were essentially similar to those observed with VP_4. On the other hand, monolayers were not formed in cultures grown in Martin and Vidler's B medium.

Growth of cells in cultures derived from trypsinized tissues

Cells suspended in medium E-V-C + 10% foetal bovine serum attached to the substrate, tube walls or coverslips, in about five hours after inoculation. In the primary cultures a lag phase of about four days was followed by active cell multiplication, monolayers resulting in about 15 days (monolayer = 25 dots on the Chalkley disc superimposed upon cells).

It is evident from the graphs (Fig. 26) that the growth rates in the subcultures were lower than those in the primary cultures. In the former, monolayers were not formed before 28 days after inoculation. Cells from the second subculture failed to grow when transferred into new medium.

In all cultures fibroblast-like cells predominated.

Histological observations on Rhipicephalus appendiculatus organ cultures

Active peristalsis, one of the parameters useful in estimating the viability of certain organs in cultures, was restricted to the gut diverticuli, Malphigian tubules and rectum. To assess the viability of those organs which did not exhibit rhythmic contractions, histological sections of entire explants cultured for 6 weeks were prepared. Those preparations were examined and compared with sections of uncultured organs taken from the same developmental stage (I + 5).

The method of preparation of the cultures and active peristalsis which occurred in such cultures caused displacement and thus changed the mutual relationships among the various organs. Consequently these relationships were quite different from those observed in comparable stages of non cultured ticks.

In the cultured explant (Fig. 27) the brain adjacent to the genital orifice, consists of an inner mass of interwoven fibres, the neuropile, and is surrounded by a layer of ganglion cells stained deeply with the haematoxylin. The entire organ is ensheathed by a thin neurilemma which is stained by the light green (Figs. 27 and 28). In a section of the cultured tissues (Fig. 28) the brain is penetrated by the oesophagous and the appearance of these two organs is very similar to that seen in comparable sections of noncultured ticks (Fig. 29). The epithelial folds with their green-staining cuticular lining and their thick muscular wall are seen in a section through a part of the female reproductive system (Fig. 30). The activity of the Malpighian tubules results in the accumulation of large guanine spherules (Fig. 31).

The gut of non-cultured ticks is filled with red blood cells many of which have fused into a homogenous mass contained within a thin epithelial layer (Fig. 29). As illustrated in Fig. 32, after 7 days in culture, the cells of the external epithelial layer, actively involved in intracellular digestion, are seen migrating into the gut lumen. This figure also shows partial digestion of the blood meal. In a section of a 6-week culture (Fig. 33) the gut lumen is seen to be completely filled with the epithelial cells. They are now highly vaculoated, some of them containing distorted nuclei and large eosinophilic globules.

Cultivation of tissues and cells of Rhipicephalus bursa, Amblyomma hebraeum and Ixodes ricinus

Rhipicephalus bursa and A.hebraeum. Stage I + 5 developing adults were placed in Leighton tubes and covered with a thin film of VP_4 medium. The cultures were incubated at 28°C and the medium changed every 7 days.

After 3 days, cells began to migrate from the explants and by the 10th day had formed monolayers over the entire surface of the coverslips. Stained preparations of 30-day cultures contained fibroblasts and small epithelial cells in all stages of mitosis (Fig. 34). Mitotic divisions were noted among the larger epithelial cells containing partially digested haemin in the cytoplasm (Fig. 35).

The cells in the Amblyomma cultures were larger than those in comparable cultures of Rhipicephalus.

Peristalsis of Malphigian tubules, gut diverticuli and the rectum was observed during the entire 30 day period of cultivation.

Ixodes ricinus. Partially engorged adult ticks were removed from bovine hosts on the Isle of Raasay. The entire body of a tick was placed in a Leighton tube, covered with VP_4 medium and incubated at 28°C. Nine ticks were used in the experiment.

After 3 or 4 days the gut in the cultures was undergoing active peristalsis and cells began to migrate from the tissues. Cell monolayers were found in 3 of the cultures after 12 days. Six cultures were discarded after 30 days when peristalsis ceased and the cells had begun to degenerate. After 6 weeks one of the remaining three cultures contained a shimmering mass of epimastigotes (Figs. 36 and 37). These flagellates were cultured for a further 2 weeks in VP_4 medium in the absence of tick tissues. The remaining two cultures were stained after 40 days when they were found to contain some dividing fibroblasts and epithelial cells (Figs. 38 and 39).

Chapter 6

Experiments with Salivarian Trypanosomes (*Trypanosoma brucei* and *T.congolense*) in Tissue Cultures of *Glossina morsitans*

Series

1 The growth of *T.brucei*.

2 Effect of the inoculum size on the growth of *T.brucei* and *T.congolense*.

3 Effect of the volume of culture medium on growth of *T.brucei*.

4 Growth of *T.brucei* and *T.congolense* during serial passage in tsetse tissue culture.

5 Growth of *T.brucei* in cultures of different types of pupal tissues.

6 Growth of *T.brucei* in cultures of the complete alimentary tract from pupae of different ages.

7 Effect of extracts of *Glossina* alimentary tract on growth of *T.brucei*.

8 Effect of medium preconditioned by incubation with the complete alimentary tract of *Glossina* on growth of *T.brucei*.

9 Growth of *T.brucei* in a system in which a semipermeable membrane separated the flagellates from *Glossina* alimentary tract.

10 Growth of *T.brucei* and *T.congolense* separated from red blood cells.

11 Growth of *T.brucei* and *T.congolense* using stabilates kept in dry ice or liquid nitrogen as inocula.

12 Morphology of trypanosomes in tsetse tissue culture.

13 The infectivity of T.brucei and T.congolense cultivated with tsetse tissues.

Conditions employed in the above series of experiments.

1. In all instances the culture medium was the modified medium of Trager (No. 9, Table 1), found most suitable for growth and maintenance of tsetse tissues and organs.

2. The hanging-drop method was used in all series, except No. 3.

3. Cultures of complete alimentary tract served as the sole system in the series 1-4, 6 and 10-13.

4. The tissues were obtained from pupae older than 21 days, except for series No. 6.

5. The volume of the growth medium was 15-20 µl, except for series No. 3.

6. The 15-20 µl, were taken from suspensions with about 1000 trypanosomes/mm^3 except for series No. 2.

7. Trypanosomes were obtained aseptically from the tail of mice infected with T.brucei (TREU 667) and T.congolense (TREU 694) except as indicated.

8. In each experiment, haemocytometer counts and stained preparations of the parasites in two or three hanging-drop cultures were made except when stated otherwise, and in each series an experiment was repeated once or twice.

9. Most of the experiments were controlled by maintaining the trypanosomes in hanging-drops of the nutrient medium without tsetse tissues. No growth of the parasites was observed in any of the control preparations in which there was an immediate decline in the trypanosome populations, and no living organisms remained after the 5th day.

Series 1: The growth of T.brucei

With an inoculum of bloodstream trypanosomes of 850-950/mm^3 there was a lag phase of about 24 hours followed by a period of logarithmic growth which lasted for about 5 days. During this time the organisms increased approximately 30 fold (Fig. 40). A stationary phase of 3 days was followed by a rapid decline of the trypanosome population until about the 14th day when only a few motile flagellates remained. The significant differences between the numbers of parasites in individual cultures are reflected in the large sample standard deviation of the daily means shown in Appendix table 2.

In this and all subsequent series in which multiplication of the trypanosomes occurred it was accompanied by morphological transformation into long slender forms similar to those seen in the midgut of the tsetse fly.

Series 2: Effect of the inoculum size on the growth of T.brucei and T.congolense

It was demonstrated in the preceding experiment that an inoculum of bloodstream T.brucei of about 1000/mm^3 resulted in rapidly multiplying parasite populations. To ascertain the effect of smaller inocula upon growth, initial concentrations of about 600, 300 and 100 flagellates/mm^3 were also employed with both T.brucei and T.congolense. Two cultures from each group were examined every 2 days for a period of 8 days.

As shown in Figs. 41 and 42, the parasites multiplied in all cultures. In those inoculated with suspensions containing 1000 trypanosomes/mm^3, maximum populations were reached by the 6th day, the peak being followed by a slight decrease in the

numbers of motile organisms. However, trypanosome populations were still increasing by the 8th day in cultures containing smaller initial inocula (600/mm^3 for T.brucei and 600, 300 and 100/mm^3 for T.congolense). The slowest growth rate of T.brucei was observed in the cultures inoculated with trypanosomes from suspensions containing 300 and 100 organisms/mm^3. In such cultures the final increase of parasite numbers was about 4 and 2 fold respectively compared with the 30 fold increase reached by this species when larger inocula were used. Inocula of T.congolense from suspensions of 100mm^3 resulted in a more rapid growth of parasites than comparable inocula of T.brucei.

Series 3: Effect of the volume of culture medium on growth of T.brucei

During previous experiments successful growth of T.brucei was observed in cultures in which the volume of trypanosome suspension in the modified Trager's medium bathing the tsetse alimentary tract was 15-20 µl. To find out whether this tsetse organ would support a similar multiplication rate of the parasites in larger volumes of the standard trypanosome suspension (about 1000 organisms/mm^3), an experiment was set up in which 100 µl, 0·5 ml and 1·0 ml of this suspension were added to individual alimentary tracts of Glossina. Hanging-drop cultures containing 20 µl of suspension were employed as controls. Sterilin tissue culture chambers were used for cultures with 100 µl of the parasite suspension and Leighton tubes for the two larger volumes.

It is evident from Fig. 43, that the growth rate of the trypanosomes was highest in the 20 µl hanging-drop cultures.

In cultures with 100 ul the numbers of organisms increased about 12 fold in the course of 5 days. Very little multiplication occurred in the Leighton tubes.

Series 4: Growth of T.brucei and T.congolense (TREU 623) during serial passage in tsetse tissue cultures.

After growing for 5 days in the presence of tsetse alimentary tract cultures, the trypanosomes were transferred to drops containing freshly dissected homologous organ cultures. The trypanosomes were passaged five times in this manner for 30 days when the experiments were terminated. The transfers were made as follows:

1. The supernatant fluid bathing the tsetse tissues and containing the trypanosomes was withdrawn from a 5-day culture.
2. This supernatant fluid was added to a drop of fresh growth medium.
3. The number of the parasites was adjusted to about 1000/mm^3 and drops of the resulting suspensions added to freshly prepared tsetse tissues and set up on Butt slides.

Trypanosoma brucei (Fig. 44). In the initial cultures a 24 hour lag phase was followed by a period of exponential growth which continued to the 5th day. No lag phase was noted during the 1st and 2nd passages, the exponential growth lasting for 5 days. However a 24 hour lag phase occurred during the 3rd, 4th and 5th passages. After the 4th serial transfer the growth rate was lower than in the preceding passages and the original cultures, the final populations increasing between 11 and 16 fold compared with over 30 fold increments in the earlier cultures.

Trypanosoma congolense (Table 7). In the original cultures there was a lag phase lasting 24 hours. Subsequently exponential growth ensued, resulting in a 30-fold increase of the parasite numbers. No lag phase was observed in any of the serial passages. The numbers of trypanosomes increased over 32-fold in the 1st and 2nd passages after 5 days, but decreased slightly to 29 or 30-fold in the following 3 passages.

It is evident from the above experiments that both T.brucei and T.congolense can be grown in the presence of Glossina tissues by serial transfer for a period of 30 days.

Series 5: Growth of T.brucei in cultures of different types of tissues from pupae aged 25-27 days

Hanging-drop cultures of the head (brain) with attached salivary glands as well as those of the abdominal wall (about 1 mm^2 explants) were used in addition to the previously employed cultures of the complete alimentary tract.

Counts made daily for a period of 5 days indicated that the highest numbers of trypanosomes were reached in the presence of complete alimentary tract (Fig. 45). The numbers were lower in cultures containing brain and salivary gland tissues and still smaller populations were obtained in preparations with body wall tissue. In the presence of all three types of tissues tested, a 24 hour lag phase preceded exponential growth. As reflected in the magnitude of the sample standard deviations (Appendix table 7), there were large differences among individual determinations, especially on day 2 in cultures containing complete alimentary tract, and brain and salivary glands. These variances were less

on the 5th day.

It is evident from the results obtained in this series that of the different tissues tested the complete alimentary tract best supported the growth of T.brucei.

Series 6: Growth of T.brucei in cultures of the complete alimentary tract from pupae of different ages.

The alimentary tracts used in this series were obtained from 7-, 14-, 21- and 27 days old pupae as well as from newly emerged flies.

As shown in Fig. 46, the highest numbers of trypanosomes were obtained in cultures with tissues from 27 days old pupae and from newly emerged flies. The growth was also quite good in the presence of alimentary tract from 21 day pupae. The poorest results by far were obtained with tissues from 7 days old pupae in which only a 4-fold increment in the parasite numbers was recorded.

Evidently tissues from older pupae support better growth of Trypanosoma brucei.

After it was established that living tissues from Glossina morsitans were essential for growth of the trypanosomes and that among the several tissues tested, the complete alimentary tract from older pupae or flies was the most suitable for the cultivation of the parasites, experiments (Series 7-9) were performed to elucidate the nature of the stimulatory effect exerted by this organ.

Series 7: Effect of extracts of Glossina alimentary tract on growth of T.brucei.

All experiments in this series were controlled by cultures

containing the complete alimentary tract. Active growth of parasites was noted in all the control cultures.

The extracts used as culture media for the trypanosomes, were prepared as follows:

(a) Alimentary tracts from 10 pupae older than 23 days were homogenised in 100 µl of the culture medium using an all-glass tissue grinder. The homogenate was centrifuged in haematocrit tubes at 10,000 g for 5 minutes. The supernatant fluid without the lipid layer was used.

(b) Alimentary tract homogenate prepared as above but without centrifugation was used and designated 'crude' extract.

Irrespective of whether or not the lipid and debris fractions were present, no multiplication of the parasites occurred when the extract served as the culture medium (Table 8). Evidently, unlike the living tissue, the extracts were incapable of supporting growth of T.brucei.

Series 8: Effect of medium preconditioned by incubation with the complete alimentary tract of Glossina on growth of T.brucei.

Several cultures of the complete alimentary tract were incubated for 3 or 4 days. The medium removed from these cultures was inoculated with bloodstream trypanosomes, incubated and sampled daily for 5 days. The experiments were controlled in two ways. One control contained the complete alimentary tract and the trypanosomes and the other consisted of non-preconditioned medium inoculated with a comparable number of parasites.

It is shown in Table 9 that no growth of the parasites was

observed in either the preconditioned or the non-preconditioned medium in the absence of living Glossina tissues. On the other hand, the trypanosomes multiplied actively in the cultures containing the complete alimentary tract.

The foregoing results indicate that preconditioning does not impart growth-promoting properties to the culture medium.

Series 9: Growth of T.brucei in a system in which a semi-permeable membrane separated the flagellates from Glossina alimentary tract.

The cultures were set up in Sterilin slide tissue culture chambers (see Materials and Methods and Fig. 2). Hanging drop cultures with and without the complete alimentary tract served as controls.

No growth of the trypanosomes was noted in drops of culture medium alone. As illustrated in Fig. 47, a similar growth pattern was observed in cultures in which the trypanosomes were separated from the alimentary tract by a semi-permeable membrane and in those in which they remained in direct contact with the living tissues of Glossina. However, the flagellates separated from the tissue by the membrane had a slightly longer lag phase and never reached final numbers as high as those in direct contact with the alimentary tract. There was a significant difference between the populations of trypanosomes cultured with and without contact with gut ($t = 4{\cdot}758$, $P < 0{\cdot}01$). Microscopic examination of the sterilin chambers, revealed that the trypanosomes multiplied in aggregates immediately beneath the tissue forming a small halo. They were seen only infrequently at the edge of these

culture drops, in contrast with the control cultures in contact with tsetse tissues in which the parasites tended to be no more numerous near the tissues than at the periphery of the hanging drops.

Although the growth of the trypanosomes was not as good in the cultures in which they were separated from the alimentary tract by a semi-permeable membrane, it is evident that the growth factors were capable of traversing the membrane. This suggests that the growth-promoting factors could be of relatively small molecular size.

Series 10: Growth of T.brucei and T.congolense in the absence of red blood cells

Most culture media used hitherto for the cultivation of Trypanosomatidae contained haemin. With Trypanosoma spp. this compound is usually in the form of red cells or red cell lysates which are considered essential for growth (Guttman and Wallace, 1964; Taylor and Baker, 1968). In the previous experiments the initial trypanosome inoculum contained red blood cells. It has, therefore, to be assumed that all subsequent passages could have contained some of these cells which provided a source of haemin. The tsetse tissue culture system offered an opportunity to test whether or not red blood cells actually constituted an absolute requirement for T.brucei and T.congolense. It is, however, realised that the foetal bovine serum used in the medium could form a source of haemin as tests have shown that samples contained concentrations ranging from 70-100 mg/100 cm^3.

Trypanosoma brucei bloodstream forms were separated from mouse red cells by centrifugation at 300 g for 5 minutes. A pellet of

the trypanosomes was obtained by spinning the red-cell-free supernatant fluid at 650 g for 5 minutes. Subsequently the parasites were suspended in Trager's modified medium to give a final concentration of about 1000 organisms/mm^3. The trypanosome suspensions were prepared as hanging drop cultures with the complete alimentary tract from tsetse pupae.

Separation of T.congolense from mouse red cells was accomplished by Lanham's (1968) DEAE anion exchange column method, using 12·5 x 1·5 cm columns, DE 52 and phosphate-saline-glucose buffer at pH 8·0. Trypanosomes obtained in the eluate were pelleted by centrifugation at 650 g for 5 minutes at 4°C. The pellet was washed twice in modified Trager's medium and hanging drop cultures of tsetse alimentary tract were set up as for T.brucei (see above).

It is evident from Table 10 that for both species the initial 24-hour lag phase was followed by rapid growth during the following 4 days, resulting in a 35- and 28- fold increase of the T.brucei and T.congolense populations respectively. After 3 days all actively growing cultures consisted of tsetse gut form-like trypanosomes.

These results indicate that red blood cells are not essential for growth of T.brucei and T.congolense in tsetse tissue cultures.

Series 11: Growth of T.brucei and T.congolense using stabilates kept in dry ice or liquid N_2 as inocula.

The numbers of trypanosomes in the blood of laboratory rodents fluctuate and thus can make the infected rodent blood an unreliable source of parasites for culture. The employment of stabilates of known trypanosome populations as sources of material for cultivation

could reduce the time taken and the use of large numbers of laboratory animals in setting up cultures. To test this alternative the following experiments were performed.

(a) A stabilate of T.brucei (TREU 667) prepared from rat blood and kept in dry ice, with 7·5% glycerol as the cryoprotectant, was added to modified Trager's medium and the parasite concentration adjusted to the usual concentration. The suspension of the trypanosomes was used for bathing tsetse alimentary tract in hanging drops cultures.

Microscopic examination 24 hours after inoculation revealed the presence of large numbers of crystals formed from infected rat blood and observed previously in the original samples of mixtures of the stabilate and culture medium (Fig. 48). These crystals, which persisted for 6 days, masked the trypanosomes and thus rendered quantitative determinations of the parasites very difficult. Although the flagellates appeared to increase in number, stabilates prepared from infected rat blood were considered unsuitable for experiments to test the value of cryobiologically preserved stabilates of T.brucei for cultivation in the presence of tsetse tissues.

(b) Stabilates of T.brucei prepared with blood from heavily infected mice and kept in dry ice, with 7·5% glycerol as the cryoprotectant, were used according to the procedure described in (a) above.

After a 48-hour lag phase, the parasites entered a period of exponential growth (Table 11). The numbers of trypanosomes counted on the 5th day correspond closely with those observed

in cultures initiated directly with blood from infected mice. Further, by using the previously described method (see series No. 4) it was possible to maintain the trypanosome cultures by serial transfers in tsetse tissues for up to 4 weeks when the experiment was discontinued.

(c) Stabilates of T.congolense (TREU 694) prepared on two separate occasions from heavily infected mice and kept in the presence of 7·5% glycerol in dry ice or liquid nitrogen were found to contain only a very few motile flagellates (one trypanosome/object field using a 25X objective and a 10X eyepiece). Trypanosomes in cultures initiated from such stabilates failed to multiply and died within two days. The stabilates were, therefore, unsuitable as a source of trypanosomes for cultivation in the presence of tsetse tissues.

(d) Blood from a mouse infected with T.congolense (TREU 261) was used, with the appropriate dilution with modified Trager's medium, as the inoculum for hanging drop cultures of tsetse alimentary tract. After 5 days, the transformed culture-form trypanosomes were frozen in liquid nitrogen (-196°C) in the presence of 7·5% glycerol and stored for 40 days. The recovery rate of the trypanosomes, as estimated by the numbers of motile flagellates, was found to be quite high in thawed stabilates. Samples of these stabilates were diluted with culture medium and prepared in hanging drops with tsetse alimentary tract.

The results of this experiment are shown in Table 12. After a 24 hour lag phase, the trypanosomes entered an exponential growth period which lasted for 5 days resulting in a 30-fold increase in the trypanosome populations.

According to the results obtained in the experiments (a) and (b), stabilates of T.brucei in mouse blood kept in dry ice are more suitable for cultivation in tsetse tissue cultures than stabilates of this species prepared from blood of infected rats. As observed in experiment (c), stabilates of T.congolense prepared from mouse blood and maintained at -72°C or -196°C contain too few blood stream trypanosomes to initiate similar cultures. On the other hand it is evident from the results of experiments (d) that the culture forms of T.congolense can be preserved successfully in liquid nitrogen and that those stabilates are suitable for cultivation with tsetse tissues in hanging drops.

Series 12: Morphology of trypanosomes in tsetse tissue cultures

In the presence of alimentary canal and salivary glands from Glossina pupae, the bloodstream forms of T.brucei and T.congolense entered a 24 hour lag phase. Thereafter they began to multiply and transform. Within 4 or 5 days the parasites multiplied to such an extent that the cultures contained a shimmering mass of trypanosomes (Figs. 49 and 50). The flagellates were evenly distributed in the hanging drops, moving freely in the culture medium. Occasionally some trypanosomes were seen attached to the tsetse tissues and the large fat cells (Fig. 51).

T.brucei: The bloodstream trypanosomes included the numerous structural forms characteristic of pleomorphic strains belonging to the subgenus Trypanozoon. The three well defined forms (Fig. 52) were: a) "long slender" organisms with a long free flagellum, a well-developed undulating membrane, an elongated nucleus, a

subterminal kinetoplast and a narrow posterior end; b) "intermediate" forms with a shorter anterior flagellum and a kinetoplast nearer to the blunter posterior end; c) "stumpy" trypomastigotes without a free flagellum, with a prominent undulating membrane, a rounded nucleus (often displaced towards the posterior end in postnuclear forms) and a kinetoplast near the broadly rounded posterior end.

Observations of 3-day cultures revealed the presence of a trypomastigote form different from those seen among the bloodstream trypanosomes. In the new form, the undulating membrane was less pronounced than in the bloodstream stages, a free flagellum was present and the kinetoplast was located about halfway between the nucleus and the posterior end of the cell. In all structural aspects these organisms resembled the midgut stages from infected tsetse flies and the flagellates typical of cultures grown in non-living media. From the 5th day on, the flagellates tended to become more slender and elongated (Fig. 53), resembling the proventricular forms found in Glossina. On several occasions cultures older than 15 days contained concentrations of trypanosomes at the periphery of the drops. The structural forms seen in such populations included the slender proventricular-like forms, large multinucleate organisms (Fig. 54) and rounded spheromastigote forms (Fig. 55).

T.congolense: The strain (TREU 694) employed in the morphological studies is typical of the species, the smallest in the subgenus Nannomonas. In the bloodstream forms (Fig. 56), a free flagellum is absent, the undulating membrane is inconspicuously developed and the kinetoplast, often in a marginal position, is situated near the blunt posterior end.

After 3 or 4 days in tsetse tissue culture, the blood-stream forms have transformed into more slender trypomastigotes, with the kinetoplast located midway between the posterior end of the body and the nucleus (Fig. 57). These organisms resembled the tsetse midgut forms. Proventricular-like trypanosomes were noted in the older cultures, which also contained a few aberrant forms.

The length of the trypanosomes was measured in bloodstream forms and on the 1st, 2nd, 3rd, 4th, 5th and 11th day of cultivation. All measurements were made on fixed and stained preparations and 100 organisms were measured on each day. The results are summarized in Tables 13 and 14.

Table 13

Length of T.brucei (TREU 667) bloodstream forms and organisms on different days of cultivation.

Age of culture (days)	Measurements (μm) Mean*	Range
0+	22·5	20-27
1	24·5	21-28
2	26·5	24-30
3	30·5	28-33
4	31·2	28-37
5	31·9	29-36
11	31·4	28-36

Table 14

Length of T.congolense (TREU 684) bloodstream forms and organisms on different days of cultivation.

Age of culture (days)	Measurements (μm) Mean*	Range
0+	15·8	13-19
1	23·2	20-26
2	26·1	23-29
3	38·0	36-40
4	42·3	35-47
5	40·8	36-43
12	40·2	36-41

(footnotes for tables on previous page)

* In all instances the mean is calculated from 100 individual measurements (Tables 13 and 14).

\+ Bloodstream forms (Tables 13 and 14).

Series 13: The infectivity of T.brucei and T.congolense cultivated with tsetse tissues.

Each day trypanosomes were harvested from 3 hanging-drop cultures and the pooled suspension was diluted in 0·2 ml IBSS. Aliquots of 0·1 ml of the resulting suspension were inoculated intraperitoneally into mice.

The results of the animal inoculations are presented in Table 15.

Table 15

Infectivity to mice of T.brucei (TREU 667) and T.congolense (TREU 694) grown in tsetse tissue cultures.

Age of cultures (days)	T.brucei	T.congolense
0*	+ (5)/	+ (7)/
1	+ (5)	-
2	+ (6)	-
3	+ (7)	-
4	+ (8)	-
5-20	-	-

* Bloodstream forms

/ Prepatent period in days

As shown in Table 15, T.brucei remained infective to mice for up to 4 days in culture, the prepatent period ranging from 5 to 8 days. None of the T.congolense cultures were infective to mice.

Chapter 7

Growth of Trypanosoma brucei (TREU 667) in Cultures of the Complete Alimentary Tract of Several Species of Arthropods

Glossina spp.

In the following experiments all the conditions were the same as those found previously to be most successful for the cultivation of T.brucei (TREU 667) in cultures of G.morsitans alimentary tract (Chapter 6). Thus the complete alimentary tracts from 25-27 day pupae of several species of Glossina were cultured in Trager's modified medium and the inocula of the trypanosomes were about 1000 blood-stream forms per mm^3 from tail blood of infected mice in 10-15 µl of medium. In all instances the pupal extracts were prepared from 10 day pupae of species homologous to those that were to be tested in a given series. The following species of Glossina were used:- G.morsitans morsitans (as the control system), G.morsitans submorsitans, G.austeni and G.palpalis. The paucity of G.m. submorsitans pupae prevented subcultures of the parasites into homologous cultures. All observations using this species were limited to the primary cultures in which growth of the trypanosomes was estimated for 5 days. With the other species of tsetse, the growth of T.brucei was studied for 10 days during which time the parasites were subcultured once on the 5th day. Three cultures were examined daily and the experiment was repeated once. All experiments were controlled by hanging-drop cultures of the trypanosomes in the absence of Glossina tissues.

As shown in Table 16, the growth of T.brucei was about the same in the presence of cultures of complete alimentary tract

from G.morsitans morsitans, G.m. submorsitans and G.austeni. Somewhat better growth was observed, however, in cultures containing G.palpalis tissues, the numbers of parasites increasing about 37-fold within 5 days in both the primary cultures and the subcultures. No growth of trypanosomes was noted in Trager's medium alone. The trypanosomes transformed into tsetse midgut-like forms in the presence of tissues from all the Glossina spp. tested.

Arthropods other than Glossina

To ascertain whether cultures of the complete alimentary tract from haematophagous arthropods other than Glossina would support growth of trypanosomes, developing adult ticks, Rhipicephalus appendiculatus and adult mosquitoes, Aedes aegypti, were used as donors of this organ system in the following experiments. The bloodstream trypanosomes obtained from tail blood of infected mice and suspended in appropriate media at a final concentration of about $1000/mm^3$ and 10 µl of the suspension. Hanging drop preparations consisting of trypanosome suspensions in appropriate media, but lacking the arthropod tissues, served as controls in all experimental series. At least two cultures were examined daily and each experiment was repeated once.

(a) Tick tissues

The entire contents of the cuticle of engorged I + 6 stage R.appendiculatus nymphs were placed in drops of either modified Trager's medium without tsetse pupal extract or in medium VP_4 + 10% foetal bovine serum.

Rhythmic contractions of gut diverticuli and of Malpighian tubules were observed during 5 days in cultures containing medium VP_4 + 10% foetal bovine serum, but not in modified Trager's medium.

No multiplication of T.brucei occurred in cultures of tick alimentary tract irrespective of the kind of medium bathing the tissues (Table 17). In the presence of tick tissues there was a gradual decline in the parasite numbers, only a few motile trypanosomes remaining by the 5th day. Even these organisms had the abnormal "tadpole-like" appearance. Without the tick alimentary tract the trypanosomes died out by the 4th day of cultivation in both growth media.

(b) Mosquito tissues

Newly emerged Aedes aegypti females, reared under aseptic conditions, were placed at 4°C for 20-30 minutes. The immobilized flies were sterilized in 10% Roccal for 10 minutes and subsequently washed three times in sterile IBSS. The entire alimentary tract, including the crop, was pulled out of a decapitated mosquito by the method employed previously with tsetse flies. After being separated from the fat body, each alimentary tract served for the preparation of one hanging-drop culture and in another experiment three explants were placed in a drop. Trager's modified medium supplemented with mosquito pupal extract and 20% foetal bovine serum, or Schneider's (1964) mosquito cell culture medium with the same serum supplement was used in this series of experiments.

In cultures bathed by both media there was active peristalsis of the alimentary tract during the 5-day period of an experiment, the mosquito tissues evidently having remained healthy. Yet the trypanosomes failed to multiply either in the presence of one or 3 explants (Table 18) per hanging-drop. Many more motile organisms were observed after 5 days in the latter system in which the population decline appeared to proceed at a much slower rate.

In all instances, however, the parasites seen on the 5th day had abnormal shapes, mostly with an enlarged, rounded posterior end. In general, the surviving trypanosomes were relatively sluggish. The numbers of the flagellates decreased more rapidly in the absence of mosquito tissues.

It is evident from the foregoing results that alimentary tract tissues from ticks and mosquitoes do not support growth of T.brucei. That the failure of the trypanosomes to grow in mosquito tissue culture does not depend on the relatively smaller volume of a single mosquito gut in comparison with that of a tsetse fly is demonstrated by the results of the experiments in which three explants per drop were used. In such cultures the tissue/medium volume ratios were approximately the same as that found in the tsetse gut hanging-drop cultures.

(c) Tissues of Sarcophaga argyrostoma

It was demonstrated by the immediately preceding experiments that tissues from some haematophagous arthropods, i.e. mosquitoes and ticks, failed to support the growth of T.brucei. In the search for a suitable substitute for tissues of the often unobtainable tsetse flies, the readily available Sarcophaga argyrostoma, which together with Glossina, belongs to the suborder Cyclorrhapha was tried as donor of the alimentary tract.

The complete alimentary tracts were obtained by the previously described method from puparia containing flies about to emerge. Before dissection the puparia were sterilized by the standard treatment with Roccal and subsequently washed in IBSS. Modified Trager's medium was employed in all instances. The medium was supplemented with an extract from 8-day Sarcophaga pupae obtained

by the usual method. The trypanosome suspension, about 10 µl per hanging drop culture, contained 800-1000 blood stream trypanosomes/mm^3. At least two cultures were examined daily and the experiment was repeated once. The complete growth medium without fly tissues was employed in control cultures.

Very active peristalsis of the midgut, Malpighian tubules and the rectum was observed during the first 5 days. An original decrease of the trypanosomes on the 1st day was followed by multiplication of the flagellates, resulting in an 18-fold increase of their numbers by the 5th and a 26-30-fold increase by the 8th day. By day 5, all the parasites were tsetse midgut-like forms. A rapid decline of the population was observed in the absence of the insect tissues (Table 19).

The experiments with Sarcophaga tissues were spoiled to a certain degree by the presence of a trypanosomatid, identified tentatively as Herpetomonas muscarum (Fig. 58), in 50% of the hanging-drop cultures. In such cultures the herpetomonads multiplied very rapidly and tended to outnumber the trypanosomes by the 3rd day, no trypanosomes remaining on the 4th day.

Chapter 8

Cultivation of Different Species and Strains of Salivarian Trypanosomes in *Glossina* Tissue Cultures

It was shown in the experimental series detailed in Chapter 6 that the pleomorphic strain of *T.brucei* (TREU 667) and the fly-transmitted strains of *T.congolense* (TREU 623 and 694) could be grown in cultures of the complete alimentary tract of *Glossina morsitans*. In view of these results, experiments were designed to test whether this culture system could support the growth of laboratory adapted strains of the above two species and other species of Salivaria. Cultivation of the tsetse fly-transmitted strain of *Trypanosoma vivax* (TREU 1101) was also attempted.

All cultures contained the complete alimentary tract of *G.morsitans* pupae over 25 days old and were prepared according to the method described previously. The following trypanosome strains, all adapted to rodents by prolonged series of passages, were employed: *T.brucei* (Etat 5), *T.congolense* (TREU 261), *T.vivax* (TREU 723), *T.gambiense* (R_3 and L_2), *T.rhodesiense* (TREU 788) and *T.evansi* (TREU 381). In addition, a tsetse fly-transmitted strain of *T.vivax* (TREU 1101) was also used.

The inocula of bloodstream trypanosomes were obtained from tail blood of infected mice except for the fly-transmitted *T.vivax* and the two strains of *T.gambiense* when stabilates were used.

In all the series, except for those of *T.brucei* and *T.gambiense* two or three cultures were sampled daily and each experiment was repeated once.

Hanging-drop cultures without tsetse tissues served as controls for all experiments.

Series 1: Trypanosoma brucei (Etat 5). No change in the number of the parasites was recorded during the first two days in culture. The numbers began to rise, however, between the 2nd and 4th day and the trypanosomes continued to multiply until day 10, the population attaining a level of about 33-fold (Fig. 59).

On day 10, the flagellates were subcultured into drops containing freshly dissected tsetse alimentary tract. As shown in Fig. 59, the organisms multiplied during the following 5 days, and at the end of this period their numbers exceeded 23 times those present in the initial inoculum. No growth of trypanosomes occurred in the absence of tsetse tissues.

The bloodstream forms of this strain of T.brucei used for the inoculum of the tsetse cultures were mainly of long slender trypomastigotes (Fig. 60). By the 6th day of cultivation, only tsetse midgut-like organisms (Fig. 61) were present. These latter forms continued to divide and were maintained for the 15-day period of the experiment.

Series 2: Trypanosoma congolense (TREU 261). It is evident from the results illustrated in Table 20 that following an initial 24-hour lag phase, the parasites multiplied rapidly, the populations increasing about 30 fold by day 5. Only midgut-like forms were observed in the cultures after three days.

Series 3: Trypanosoma vivax (TREU 65 and TREU 1101). In this series, involving a rodent-adapted (TREU 65) and a fly-transmitted (TREU 1101) strain, cultures of both tsetse alimentary tract and mouthparts with attached salivary glands were used for growing the parasites. To ascertain the effect of the source of the serum on the growth of the rodent-adapted strain, the standard medium supplemented with foetal bovine serum was employed in one experiment and mouse serum (20% heat-inactivated) was incorporated into the medium used in another experiment (Table 21). Some of the cultures of TREU 1101 were incubated at 28°C and others at 31°C (after Trager, 1959) (Table 22).

In all cultures containing tsetse tissues bathed in media with foetal bovine serum or mouse serum, as well as in those without tsetse tissues, the numbers of healthy-looking trypanosomes of both strains declined after the first 24 hours. A further decrease of the populations was noted until the 4th or the 5th day when only very few or no motile flagellates remained. During this period the parasites became progressively more sluggish and most of them assumed irregular shapes, characterized typically by swollen posterior ends which imparted to them a "tadpole-like" appearance. The temperature of incubation of strain TREU 1101 did not noticeably affect the results.

Series 4: Trypanosoma rhodesiense (TREU 788). Bloodstream trypanosomes from infected mice were cultured in modified Trager's medium with alimentary tract from pupae older than 24 days. The initial inoculum was about 1000 organisms per mm^3. Two cultures were sampled daily and the parasites were grown for 10 days having been subcultured on the 5th day into drops

containing freshly dissected tsetse tissues. Control cultures without tsetse tissue were also prepared.

In the presence of tsetse tissues the trypanosomes entered a rapid phase of growth after 24 hours reaching a 29-fold increase on the 5th day (Table 23). The sub-cultures also grew well, reaching a 30-fold increase in the parasite populations by the 10th day. In the medium alone the numbers of trypanosomes decreased gradually until the 5th day when no organisms could be seen.

The blood forms were characterized by pleomorphism typical of the brucei-sub group trypanosomes. By the 3rd and 4th days in culture all the trypanosomes had transformed into long slender forms morphologically similar to those found in the midgut and proventriculus of an infected tsetse fly.

Series 5: Trypanosoma gambiense (strains L_2 and R_3). The results of the experiments with this species are shown in Table 24. During the first 48 hours there was a sharp decline in the numbers of parasites belonging to both strains. In the presence of tsetse gut, strain L_2 remained at the same level for the 10-day period of the experiment (Table 24). Only very few organisms appeared to have transformed into midgut-like trypomastigotes, the majority showing signs of degeneration. Numbers of trypanosomes belonging to strain R_3, however, increased slightly on days 6 and 8, but diminished again by day 10. Numerous midgut-like forms, as shown in Fig. 62 were seen in cultures of this strain after 6 days.

Series 6: Trypanosoma evansi (TREU 381). A rapid decline in

numbers was noted in cultures with and without tsetse tissues, the longest survival occurring in the presence of the alimentary tract tissues (Table 25). No living trypanosomes remained, however, in any of the cultures after 4 days.

Chapter 9

Growth of Trypanosoma brucei and Trypanosoma congolense in culture media with amino acid composition based on the analysis of Glossina morsitans haemolymph

In the culture medium used for the cultivation of tsetse fly tissues in the course of the studies reported in the previous sections of this thesis, lactalbumin hydrolysate presumably served as the main source of amino acids. Knowledge of the chemical composition of haemolymph has served as a valuable guide in designing several successful media for the growth and maintenance of arthropod tissues and cells (Wyatt, 1956; Schneider, 1964; Rehacek and Brozostowski, 1969; Schneider, 1971). In view of this, an analysis of free amino acids of the haemolymph of G.morsitans was carried out to develop a medium with a more defined amino acid constitution.

Amino acid analyses

Haemolymph from 10-day pupae, pre-emerged flies and 2-day old flies was retrieved and analysed according to the techniques detailed in the "Materials and Methods" (Cunningham and Slater, 1974).

The results of the amino acid analyses of the haemolymph of pupae and adult tsetse flies are summarized in Table 26 and Fig. 63. The total concentration of the amino acids present increased from 1756 mg/100 ml in the pupal haemolymph to 2021 mg/100 ml in the pre-emerged flies. This concentration decreased, however, to 1481 mg/100 ml in the 2-day old flies.

There was considerable variation in the levels of individual

amino acids among the three developmental stages. This was particularly marked for taurine, glutamine, glutamic acid, proline, alanine, tyrosine, lysine, histidine and arginine. Most of the amino acids were found in greater concentrations in pupal haemolymph than in that of the pre-emerged and 2-day old flies. However, the levels of glutamine, proline, tyrosine, phenylalanine and aspartic acid were higher in one or both of the two older stages. No aspartic acid could be detected in the pupae. Concentrations of most of the amino acids were similar in the haemolymph of the pre-emerged and 2-day flies. Exceptions to this were glutamine, proline, valine and tyrosine found in lower concentrations in the older flies. On the other hand these flies had significantly more alanine. A striking feature was the high concentrations of proline, particularly in the pre-emerged flies in which it reached a level of 1088 mg/100 ml.

Among the non-identified components eluted from the column were considerable quantities of acidic material, eluted before taurine and presumably containing some cysteic acid. A small amount (0·14 mM) α-amino butyric acid was also present.

Growth of trypanosomes in Media No. 1 and No. 2

Trypanosoma brucei (TREU 667). Medium No. 1 (see Appendix for its composition) was used for the cultivation of this species and consisted of amino acids in concentrations based upon the analysis of haemolymph of 10-day old pupae. In addition it contained inorganic salts, organic acids and carbohydrates as in the modified medium of Trager, a vitamin B complex mixture and 20% foetal bovine serum. The pH of the medium was adjusted

to 6·7 by the addition of 1·0N NaOH.

Hanging drop cultures with and without the complete alimentary tract from 26-day old tsetse pupae were inoculated with 15 µl of bloodstream trypanosome suspension in Medium No. 1 containing 1000 organisms/mm^3. Daily microscopic observations of the cultures revealed transformation to culture forms and rapid multiplication of the trypomastigotes. Haemocytometer counts on the 5th day indicated that the populations had increased about 32-fold. Trypanosomes in the cultures in medium alone died after 3 days. Upon subculture on the 5th day the trypanosomes continued to multiply at a rate similar to that noted in the primary cultures.

Trypanosoma congolense (TREU 694). Medium No. 2 (see Appendix) was used for the cultivation of this species. The amino acid composition was based on the levels which occurred in the haemolymph of 2-day old tsetse flies, except that the water solubility of some of the acids made it necessary to adjust their final concentrations. The inorganic salts, organic acids, carbohydrates and vitamin B complex incorporated in the mixture were similar to Grace's (1962) medium for silkworm cell cultures. The pH was adjusted to 7·4 by the addition of 10% KOH and the medium was supplemented with 20% foetal bovine serum.

Bloodstream trypanosomes from infected mice were suspended in Medium No. 2 and their concentrations adjusted to 1000 organisms/mm^3. Two kinds of hanging drop cultures were prepared. Some contained the usual explants of the alimentary tract from 25-day old pupae, bathed in 15 µl of the trypanosome suspension; others consisted of similar aliquots of the

trypanosome suspension alone.

Daily microscopic examination made during the first 5 days of cultivation revealed transformation and rapid multiplication of the parasites in the hanging-drops containing tsetse tissues, but not in those without the tissue explants. On the 5th day each of the six positive hanging-drop cultures was transferred into 0·4 ml of Medium No. 2 in a Leighton tube. Three tubes contained mouthparts and salivary glands from two adult Glossina; the remaining three tubes contained medium alone.

An increase in numbers of the culture trypomastigotes of 25-fold was observed in the presence and absence of tsetse tissues and a very rapid multiplication of the flagellates necessitated serial transfers every 3 days.

On the 20th day of cultivation, a change was observed in the trypanosome populations. Many of the parasites were shorter and moved more rapidly than the typical culture forms. Some of them had a blunt posterior and a tapered anterior end, and their kinetoplast was nearly terminal (Fig. 64). In many respects these flagellates resembled, in their morphology at least, the forms found in the mouthparts of infected flies and in the bloodstream of mammalian hosts (Fig. 65).

Intradermal and subcutaneous inoculations of the short forms into mice failed to result in parasitaemias, the animals remaining negative for trypanosomes during the subsequent 40 days.

Chapter 10

Growth of stercorarian trypanosomes in arthropod tissue cultures

The following stercorarian species: Trypanosoma (Herpetosoma) musculi, T.(Herpetosoma) lewisi, T.(Megatrypanum) theileri and T.(Megatrypanum) melophagium were grown in hanging-drop cultures of the complete alimentary tract from Glossina morsitans morsitans pupae older than 23 days. Trypanosoma musculi was also grown in cultures of the gut from the I + 6 stage (6 days after immobilization) of developing adults of Rhipicephalus appendiculatus. Trager's modified culture medium with pupal extract was used for the cultivation of tsetse tissues, while VP_4 + 10% foetal bovine serum served for the tick tissue cultures. The experiments were controlled by growing the parasites in hanging-drops of the appropriate culture medium in the absence of arthropod tissues. All cultures were incubated at 28°C and at least 2 cultures were sampled daily.

Series 1: Trypanosoma musculi (TREU 1094)

Trypanosomes from tail blood of heavily infected mice were used for inoculating the arthropod tissue cultures. Before inoculation the numbers of parasites were adjusted with the appropriate medium to between 850 and 1000 organisms per mm^3. The organisms grown with and without arthropod tissues were subcultured every 4 or 5 days.

To test the infectivity of the trypanosomes to mice, the fluid from three hanging-drop cultures with arthropod tissues and from three without those tissues was pooled daily. Sufficient volumes of IBSS was added to each pooled suspension

to give 0·4 ml, and 0·2 ml samples were inoculated intraperitoneally into each of two mice.

Growth with tsetse alimentary tract. After 24 hours there was a decrease in the numbers of organisms, all of which resembled the bloodstream forms (Fig. 66) present in the original inocula. At this time, numerous small clusters or rosettes of small trypanosomes, which were difficult to count in a haemocytometer, were present. In the course of the following 3-4 days the clusters (Fig. 67) increased in size until the coverslips were nearly covered by a monolayer of the flagellates. Since the clusters could not be dispersed mechanically with the aid of a rubber policeman nor enzymatically by using 0·25% trypsin, quantitative estimation of growth was impossible.

Growth in Trager's modified medium without tsetse alimentary tract. The trypanosome growth, although less luxuriant, was similar to that observed in the presence of tsetse tissues.

Growth with tick alimentary tract. Transformation of bloodstream trypomastigotes into small epimastigote stages occurred within 24 hours. The original small clusters of the latter forms enlarged until the parasites covered the entire surface of the coverslip. In all respects, the epimastigotes resembled those observed in tsetse alimentary tract cultures.

Growth in VP_4 + 10% foetal bovine serum without tick tissues. The trypomastigote to epimastigote transformation and growth were also observed in the absence of tick tissues. There was,

however, less growth under these conditions than with the alimentary tract.

Morphology of the trypanosomes in the cultures. In all cultures the bloodstream forms changed into epimastigote stages (Fig. 68), resembling those found in the insect midgut and rectum. No bloodstream forms remained after a few days. Some of the flagellates were swimming freely in the medium, but most of them were attached to the surface of the coverslip. In addition to the epimastigotes, many organisms had a posterior (postnuclear) kinetoplast and a median nucleus, resembling the metacyclic trypomastigotes which occur in natural conditions in the rectum of the vector. The great variety of forms present in cultures after 7 days is illustrated in Fig. 68.

Infectivity of cultures to mice. The results of the infectivity tests of trypanosomes cultivated in tsetse and tick tissue cultures are summarized in Tables 27 and 28. Cultures in which most trypanosomes still were bloodstream forms caused parasitaemias with prepatent periods of 7 or 8 days. Subsequently most cultures were non-infective for up to the 4th day. From the 5th day onward most of the cultures with tsetse and tick tissues regained their infectivity and remained infective up to the 28th and 17th days respectively, at which time the experiments were terminated. The prepatent periods ranged from 5 to 11 days with infections from cultures containing tsetse tissues and 8 to 12 days in those initiated by cultures grown in the presence of tick alimentary tract. Trypanosomes cultivated in media alone also produced parasitaemias

in a certain number of mice. Infectivity however, was not observed in such cultures older than 8 days. It was less consistent among cultures grown in VP_4 + 10% foetal bovine serum than in Trager's modified medium (c.f. Tables 25 and 26).

During the first few days of patent parasitaemias, the bloodstream forms found in the mice infected with T.musculi included all the reproducing stages characteristic of natural infections with this trypanosome species. Some of these stages are shown in Fig. 69.

Series 2: Trypanosoma lewisi (TREU 726) in tsetse tissue cultures.

Blood from infected young rats of about 40 gm weight served for inoculations of cultures of complete alimentary tract of G.morsitans pupae in modified Trager's medium or of hanging drops of the medium alone. The cultures were maintained for 28 days by serial passages with or without tsetse tissues.

For infectivity tests, the trypanosomes from three cultures grown with and from three grown without tsetse gut were pooled daily during the first 10 days and every 4 days thereafter. Each of the two pooled samples was diluted with IBSS to a total volume of 0·5 ml, and 0·25 ml of the suspension was inoculated into each of two young rats.

Growth characteristics. In the presence of tsetse tissues, after 24 hours the bloodstream trypomastigotes (Fig. 70) transformed into epimastigote stages which formed small rosettes

containing four to six organisms. After 5 days the clusters became quite large (Fig. 71). The epimastigotes, which closely resembled the organisms found in the natural insect vector, continued to multiply until the 26th day when the experiment ceased. In Trager's modified medium alone, the numbers of epimastigotes began to decrease on about the 10th day of cultivation. Difficulties with dispersing the clusters of flagellates in both kinds of culture precluded quantitative studies.

Infectivity to rats. The results of the infectivity tests are summarized in Table 29. The shortest prepatent periods, 4 and 5 days, were recorded from rats inoculated with bloodstream forms. These periods ranged from 9 to 14 days in animals infected with the epimastigotes present in 24 hours and older cultures. Organisms cultivated with tsetse tissues retained their infectivity for the 26 day period of the experiment. During the first 3 days such cultures appeared to be less infective than those older than 4 days. Trypanosoma lewisi cultivated in modified Trager's medium without tsetse gut lost their infectivity to rats after 10 days. The dividing flagellates typical of early infections in rats inoculated with cultured parasites are shown in Fig. 72.

Series 3: Trypanosoma melophagium (TREU 89) in tsetse tissue culture.

The trypanosomes, obtained from cryopreserved stabilates, were cultivated for 5 days on blood agar slopes (Wells, 1969) at 28°C before being used in experiments. By this time

extensive growth produced large populations. The parasites were suspended in Trager's modified medium and their numbers adjusted to 950-1000 per mm^3. This suspension was used for the preparation of hanging-drop cultures with and without the complete alimentary tract from Glossina morsitans pupae older than 24 days. Three of the two types of cultures were prepared and examined daily, and the flagellates were subcultured serially in the homologous systems on days four and eight. Quantitative studies of growth were discontinued after 11 days. The entire experiment was repeated once.

Growth characteristics. As shown in Table 30, the parasites multiplied rapidly in the primary cultures containing gut, reaching a 30-fold increase in numbers by the 4th day. A similar growth pattern was noted in the subcultures. By the 10th day of cultivation the morphology of the parasites began to change and quantitative studies were terminated. A t test applied to these results showed that the difference between the growth of the trypanosomes in the presence of tsetse tissues and in medium alone was highly significant ($P < 0{\cdot}001$).

Morphology. The organisms inoculated into all hanging-drop cultures were long epimastigote forms, up to 40 μm long, with the kinetoplast located to the side or slightly anterior to the central nucleus (Fig. 73). The flagellates which continued to multiply in the cultures retained the epimastigote form until the 9th or 10th day, at which time shorter epimastigote forms began to appear in the cultures containing tsetse tissues. These shorter organisms (Fig. 74), up to 10 μm in length resembled those found in the hindgut and rectum of the natural arthropod

vector, the sheep ked. The short epimastigotes aggregated into large clusters (Fig. 75). The tendancy for clumping was particularly noticeable at the periphery of the culture drops.

In the absence of tsetse tissues, the long organisms did not transform into the shorter "hindgut-like" epimastigote forms.

Series 4: Trypanosoma theileri (TREU 641 and TREU 644) in tsetse tissue cultures.

The parasites used for hanging drop cultures with and without the complete alimentary tract of Glossina were obtained from two stabilates, TREU 641 and TREU 644 prepared from long-term cultures maintained on blood agar slants (Wells, 1969). The cultures were examined daily for the first 10 days; thereafter examinations were carried out every 3 days for a period of 3 weeks.

Growth characteristics. In the presence of tsetse tissues rapid multiplication of flagellates derived from TREU 641 stabilate resulted in a 33-fold increase of their numbers within 3 days (Fig. 76). Comparable growth was obtained in the subcultures up to the 10th day when structural changes of the parasites were observed and the haemocytometer counts were discontinued.

In Trager's medium alone, the multiplication of TREU 641 derived trypanosomes proceeded at a significantly lower rate, the population increase being about 16-fold after 3 days.

Less rapid growth was noted in primary cultures using

organisms obtained from the TREU 644 stabilate. In the presence of tsetse alimentary tract cultures a 20-fold increment in the numbers of parasites was recorded after 5 days. The subcultures, however, increased about 28-fold by the 9th day. In Trager's modified medium alone the population of organisms increased only about 9-fold by the 5th day in the primary cultures. In the latter conditions a 19-fold increase was observed after the subcultures had been grown for 9 days (Table 31).

Morphology. The parasites introduced into the tsetse tissue cultures were 35 (27 - 42) μm long epimastigotes, with a kinetoplast situated on one side of the nucleus (Fig. 77). After 5 or 6 days of cultivation, the trypanosomes became slightly shorter, with a mean of about 30 μm. In these latter organisms the kinetoplast was typically anterior to the nucelus. These shorter epimastigote forms were of variable shape, some being pear-shaped with pointed posterior ends. Further morphological changes occurred on days 9 or 10. In addition to the relatively long free epimastigote stages, many small organisms forming small clusters firmly attached to the coverslips were observed at this time. When examined in Giemsa's-stained preparations, the small organisms were about 3-4 μm long with inconspicuous flagella. Various forms of the parasites found in tsetse tissue cultures are illustrated in Fig. 78.

Chapter 11

DISCUSSION

Arthropod tissue culture

Glossina morsitans

A variety of arthropod cells, tissue and organs can be grown and maintained in many kinds of media (Jones, 1966). Some of these, for example Grace's (1962a) GMA medium designed originally for lepidopteran cells and based on the amino acid composition of silkworm haemolymph (Wyatt, Loughheed and Wyatt, 1956) have been shown to be very suitable for the cultivation of cells from other insect species (Hink, 1972). Others, among them that of Martin and Vidler (1962) for tick tissues, were developed from mixtures capable of supporting growth of mammalian cells, for example Eagle's (after Eagle, 1955). The latter media are readily available from commercial sources and this is a considerable advantage. However, Eagle's medium was found unsuitable for the cultivation of tsetse tissues, and negative results were also obtained with these tissues in VP_4 medium which successfully supports tick tissues *in vitro*.

Only a few of the media tested in the course of this study were found suitable for the cultivation of *Glossina* organs and tissues (see Tables 2 - 4). These were certain modifications of Trager's (1959) and Jones and Cunningham (1961) media. In all instances foetal bovine serum and either untreated or heat-treated tsetse pupal extracts were indispensable for the maintenance of peristalsis in organ cultures and for cell migration from tissue explants. Better results in terms of

these two parameters commonly used in estimating success of cultivation of arthropod tissues were obtained with untreated than with heat-treated extracts. This suggests that heat destroys some of the essential growth factors present in the extracts. The most spectacular results were achieved with the modification of Trager's medium (Cunningham, 1973) designated as medium No. 9, which included certain reducing agents i.e. ascorbic acid and reduced glutathione, capable of inhibiting the toxic polyphenol oxidase system present in the pupal extract. Indeed, this was the only medium in which cellular outgrowths from certain tsetse tissues contained cells in mitosis.

Although outgrowths were seen in explants of all tissues cultured in medium No. 9, mitoses were limited to those of the proventriculus. Evidently this medium, which favoured peristalsis of organs and outgrowths from the different tissues tested, supported mitosis in proventricular explants alone. The ability of the proventricular cells to divide can probably be ascribed to some unique properties of their metabolism. This ability suggests that these cells may be suitable for producing monolayer cell cultures.

Explants of most G.morsitans tissues gave rise to outgrowths which closely resembled those described by Trager (1959) from cultures of tissues of G.palpalis. This similarity did not extend, however, to the explants of G.morsitans midgut which extruded large vesicles. The different behaviour of the midgut explants can perhaps be explained by the differences in

the fly species, the age of the tissues, or the exact composition of the media employed by Trager and in the present study.

It is of interest that the vesicles extruded from the midgut explants of G.morsitans pupae were similar to those reported previously from cultures of embryonic tissues of leafhoppers (Mitsuhashi and Maramorosch, 1964), regenerating leg of the nymphal cockroach in vitro (Marks, 1968) and explants of embryonic tissues of Lepidoptera (Sohi, 1967). Vesicles formed in cultures of larval mosquito tissues were used for the establishment of cell lines (Singh, 1967; Schneider, 1969; Varma and Pudney, 1969). However, no similar attempts have been made to date with Glossina midgut vesicles.

Tick tissue and organ cultures

Undifferentiated or developing tissues and organs obtained from embryos or other immature stages provide the most suitable material for cell cultures (Paul, 1970). Such tissues usually contain many dividing cells, which are often capable of proliferation in vitro. It was observed in the course of the present study that the hypodermis (mainly epidermal tissues) from developing adult stages I + 2 to I + 7 of Rhipicephalus appendiculatus was rich in mitoses. The absence of actively dividing cells in similar tissue from older developmental stages of this tick species suggests a more advanced state of differentiation of these tissues. The suitability of the earlier stages for the initiation of cell cultures was confirmed by the finding that monolayers were formed in a large proportion of cultures started with tissues derived from stages I + 3 to I + 5, but not from those of stages older than I + 7 which were

in more advanced states of differentiation. On the other hand the difficulty in obtaining monolayers from I + 2 stage could be explained by the fragility of the gut which often resulted in its rupture and spilling of its contents over the coverslip in the culture tubes. However, all cultures derived from this stage which were set up without rupturing of the gut gave rise to monolayer cultures.

The results reported in this thesis are generally comparable with those obtained in the less detailed and quantitative investigation of Martin and Vidler (1962) who cultivated tissues from the same tick species. According to these workers extensive cell migration occurred in cultures of tissues from stages 4-7 days after engorgement. Cultivation of explants from later stages was said to be less successful, the cells degenerating after a few days in vitro. An exact comparison of the present results with those of Martin and Vidler is difficult owing to the less accurate definition of the developmental stages employed by them, for it is known that metamorphosis (immobilization) and development of adult tissues proceeds at different rates within a single group of engorged nymphs.

Whereas only tissues obtained from younger stages gave rise to cell cultures, successful organ cultures could be prepared from stages I + 3 to I + 9. The poor results observed with the I + 2 stages were caused by the difficulties described previously, and experience has shown that healthy organ cultures can be obtained only from undamaged organs. In all successful preparations the gut diverticuli and

Malphigian tubules were seen lying free over the coverslips. Among the organ cultures derived from stage I + 11, peristalsis was seen in only 50% of the preparations. It might have been present in the others but could not be observed because the organs were ensheathed by the well developed adult epidermis. It can be assumed that if the epidermis could be served to release the alimentary tract, peristalsis would have occurred also in cultures of the older stages. The observation that successful organ cultures could be achieved from stages no longer capable of initiating cell cultures suggests that the former are less dependent upon the state of tissue differentiation.

The type of growth of the cell monolayers of R.appendiculatus was unlike that characteristic of monolayers in monotypic cell cultures derived from tissues of other arthropods or of vertebrates. The tick monolayers resembled, however, those observed frequently in vertebrate liver cell cultures. Like the latter, they contained aggregates of piled up cells, often with a central cell-free zone. The monolayers seen in cultures of tissues from Ixodes ricinus, Rhipicephalus bursa and Amblyomma hebraeum, however, did not contain the large cell aggregates. The difference between the growth in tissue cultures from R.appendiculatus and in those from the other species might have depended upon species differences. Moreover, the cultures from I.ricinus used in the present study were derived from partially engorged adults in which the epidermal tissues were in a different state of differentiation from that of these tissues in the fully engorged nymphs/developing adults employed

for cultures of the other species used.

Of the three media used, VP_4 and E-V-C were found to be more suitable for the long term cultivation of tick tissues than Martin and Vidler's medium (1962). This medium consisting of Hanks' BSS, Eagle's amino acid and vitamin mixtures and 20% ox serum failed to support growth of cultures up to 100 days. It seems likely that lactalbumin hydrolysate, BP albumin and foetal bovine serum present in VP_4 and E-V-C culture media were responsible for their improved growth-promoting properties.

In the conditions used in the experiments reported in this dissertation, cell suspensions of trypsinized epidermal tissues from developing adults developed into monolayers in 15 days in primary cultures. In subsequent subcultures this period was between 20 and 28 days, and no growth occurred after the second subculture. By modifying the medium and/or the technique, it might be possible to establish a cell line of the fibroblast-like cells which predominate in the cultures. The cell types observed in the cultures prepared in the course of the present investigation resembled those described from cultures of Hyalomma dromedarii (Rehacek, 1965b; Varma and Wallers, 1965); Dermacentor andersoni (Yunker and Cory, 1965, 1967) and Rhipicephalus appendiculatus (Varma and Pudney, 1973). According to Varma and Pudney (1973), trypsinized tissues of the last species could be maintained through at least three subcultures up to 113 days when the cells began to degenerate. The cultures prepared in the present studies compared with those of Varma and Pudney in that the cell numbers diminished on successive subcultures.

The cell types seen in the cultures of the several tick species used in these studies and those observed by previous workers in similar preparations (see references listed in the preceding paragraph), were as follows: a) Large fibroblast-like cells containing dark cytoplasmic granules. Such cells were also described by Martin and Vidler (1962) from cultures of R.appendiculatus. Those workers postulated that the granules were the result of the phagocytic activity of the cells in vitro, and not necessarily indicative of a relationship to gut epithelium as they suggested that they were dedifferentiated cells of midgut epithelial cell origin. b) Smaller fibroblast-like cells, probably of either connective tissue or haemocoel origin. Tick haemocytes are known to be represented by a variety of cell types (Balashov, 1972), and arthropod haemocytes have been successfully adapted to in vitro cultivation (see various authors in E. Weiss, ed., Current Topics in Microbiology and Immunology, 1971; Cory and Yunker, 1971). c) Fibroblasts, with eosinophilic granules, seen mainly in older cultures. These granules might have been products associated with the ageing process of the cells in which no mitoses were observed. d) Very large epithelial cells, presumably derived from the midgut. These cells were filled with food globules, vacuoles and other cytoplasmic inclusions. It cannot be determined with certainty whether haemoglobin present in their cytoplasm was absorbed before or after migration of the epithelial cells from the explanted gut tissues. Since small amounts of the gut contents were often released during preparation of the cultures the second

alternative appears likely. It was suggested by Martin and Vidler (1962) that since the epithelial cells had prolapsed through the outer layer of the midgut wall, they might have provided a possible means whereby Theileria and other protozoa gained access to the tick's body cavity where they could be picked up by haemocytes and transferred to the salivary glands.

In cultures of R.appendiculatus peristalsis of the gut diverticuli, Malphigian tubules and rectum persisted for 4-6 weeks providing evidence of the healthy state of the culture. This state was reflected also in the appearance of histological sections. Examination of the gut diverticuli after 6 days and 6 weeks of cultivation revealed changes associated with digestion of the blood meal which were closely comparable to those reported from the organs of fed ticks (Till, 1961; Arthur, 1962; Balashov, 1972). Large quantities of guanine were concentrated in the rectum which was enlarged into a massive white area overlying part of the gut. Guanine was retained in the rectum and was never expelled into the culture medium. The normal function of the Malphigian tubules was manifested by the accumulation of large guanine sphericles which imparted a milky white appearance characteristic of the healthy condition of this organ. The digestive tract of insect vectors, predominantly Diptera, in culture has been the subject of many studies in which a suitable substrate for parasites was sought. In most cases the successful survival of the organs was estimated by their contraction (for review and pertinent references, see Demal and Leloup, 1972). Presently, however, only few reports are

available dealing with histological investigations of the organ cultures. The salivary glands in cultures initiated from I + 5 developing adult R.appendiculatus adults consisted of a main duct, about 38 µm in diameter with slightly swollen tips of the developing branches. However, as proved by examination of histological sections there was no differentiation into alveoli. Development of the gonads was also lacking. The absence of development of the salivary glands and gonads might have depended upon the lack of growth and differentiation hormones in the in vitro system, for, as pointed out by Marks (1970) and Demal and Leloup (1972), the presence of exogenous hormones can stimulate the development of those organs in culture.

Luxuriant growth of epimastigote trypanosomatid flagellates was noted in a few of the organ cultures of complete body contents of partially engorged Ixodes ricinus adults. These ticks were removed from bovine hosts in an area from which Trypanosoma theileri has been reported (Wells, 1969). It seems possible, therefore, that the flagellates might have belonged to this species. There are several records of T.theileri from the haemocoel of other ticks, Rhipicephalus pulchellus and Boophilus decoloratus (Burgdorfer, Schmidt and Hoogstraal, 1973). The long lag phase in the development of the trypanosomatids in I.ricinus cultures is difficult to explain. It seems reasonable to assume that if the flagellates were present in the haemocoel of the ticks and then attached themselves to the external surfaces of the explants they would appear in the cultures much sooner than if they were contained within the gut. The fragile nature of the gut

in vitro would allow the ultimate escape of the trypanosomatids into the culture medium. Thus the evidence points to the gut as the probable site from which the protozoa were derived. Of interest in this connection is the report by O'Farrell (1913) of *Crithidia* (syn. *Blastocrithidia*) *hyalommae* in the haemolymph and tissues of *Hyalomma aegyptium*. It was suggested subsequently by Wenyon (1926) that the epimastigotes seen by O'Farrell actually were stages of *T.theileri* and this view was accepted by Burgdorfer, Schmidt and Hoogstraal (1973). The present observations of the trypanosomatids in organ cultures of *I.ricinus* are too limited to allow the assignment of the protozoa to either genus. In any case, no trypanosomatids have previously been reported from the latter tick species.

Trypanosomes in tsetse fly tissue cultures

It has been customary to grow salivarian trypanosomes in several media which do not contain multiplying vertebrate or invertebrate cells or tissues (for review see Bishop, 1967). Most of these media are diphasic, and all contain either intact red cells as in NNN (Nicolle, 1908) or Tobie's (Tobie, van Brand and Mehlman, 1950) or red cell extracts (Pittam, 1970; Dar, 1971). In the more recent investigations aimed at the development of semi-defined media, red cells or their extracts have been substituted by haemin (Cross and Manning, 1973). Only a few reports are available on the cultivation of Salivaria in the presence of mammalian (Demarchi and Nicoli, 1960; Le Page, 1967; Hawking, 1971), avian (Fromentin, 1961), and insect (Trager, 1959; Nicoli and Vattier, 1964; Cunningham, 1973) cell or tissue cultures.

It is possible that, irrespective of the system for in vitro growth of the trypanosomes, haemin constitutes a nutritional requirement (see a subsequent section of this discussion).

Although useful in providing material for physiological and biochemical investigations, most of the systems have been unsuitable for studies aimed at reproducing in vitro the developmental stages of the salivarian trypanosomes found in the invertebrate vectors. Only tissue cultures of Glossina spp. have given promise as suitable systems for the latter studies. Trypanosoma brucei, T.congolense and T.vivax were grown successfully in cultures of alimentary tract and salivary glands from G.palpalis, but only T.vivax was found capable of completing its cycle under these conditions by producing forms infective to sheep (Trager, 1969 b). Trypanosoma brucei and T.congolense were also said to produce forms morphologically similar to the metacyclic stages. These forms, however, were not infective to rodents. Using cultures of similar tissues from G.fuscipes quanzenis, Nicoli and Vattier (1964) observed growth of procyclic forms of T.rhodesiense; no infective forms were produced. Neither Trager nor Nicoli and Vattier attempted to quantify their data. On the other hand the results reported previously by Cunningham (1973a, b) and those detailed in the present thesis are expressed in quantitative terms as far as is technically possible.

As reported above, hanging-drop cultures of complete alimentary tract of G.morsitans morsitans pupae older than 21 days were found to support excellent growth of T.brucei for up to 5 or 6 days in Trager's medium in which sheep serum was replaced by foetal bovine serum. The subsequent precipitous decline in the numbers of

motile organisms might have been caused by the depletion of metabolites in the small drops of medium. The nutrients would be utilized by the parasites as well as by the tsetse tissues. This assumption is supported by the finding that on serial subcultures into drops with fresh pupal tissues both T.brucei and T.congolense continued to multiply reaching about a 30-fold increase in population in each passage. Growth of this magnitude compares favourably with that recorded for T.rhodesiense and T.gambiense in Weinman's monophasic blood agar medium (Weinman, 1946). His cultures initiated with 100 trypanosomes, reached populations of about 3×10^6 within a few weeks, but no growth curves of these studies were published. Fairly rapid growth of T.rhodesiense is known to occur also in Tobie's diphasic medium. After seven days, initial inocula of 5×10^5 organisms/ml resulted in populations of $1 \cdot 4 \times 10^6$/ml, this growth being followed by a rapid decline of healthy organisms (Tobie, von Brand and Mehlman, 1950). In the entirely liquid medium of Pittam (1970), numbers of T.rhodesiense were reported to have increased from 3×10^6 to 3×10^7 flagellates/ml in 4 days. According to the recent findings of Cross and Manning (1973), no growth of two different strains of T.brucei occurred for over 15 days in a slightly modified Pittam's medium. When the culture forms were transferred from this medium to a blood-free semisynthetic mixture, reliable growth could not be obtained with inocula of less than 5×10^5 organisms/ml. Although the tsetse tissue culture system is not directly comparable to that used by Cross and Manning, small inocula of 100-300/mm^3 of T.brucei and 100/mm^3 of T.congolense,

were found less suitable than larger ones up to 1000 organisms/ mm^3, for the establishment of rigorous cultures of the parasites. Nonetheless, in cultures initiated with the smaller inocula, the numbers of trypanosomes increased very gradually, the yield being quite low on the fifth day. It is possible, therefore, that if the cultures were maintained for longer periods in the presence of healthy tsetse fly tissues, multiplication of the flagellates would continue, the populations ultimately reaching a size equal to that characteristic of 5-day cultures started with the larger inocula.

Only few published reports have dealt with the cultivation of salivarian trypanosomes in the presence of vertebrate cell cultures (see the appropriate review section of the present thesis), and still fewer contain quantitative data on the growth of the trypanosomes in these systems. According to Demarchi and Nicoli (1960), in the presence of HeLa or HEP cell cultures, populations containing 800-2000/ml of T.rhodesiense or T.gambiense resulted from inocula of 30 organisms/ml in about 6 days. Using tsetse tissue cultures grown in Trager's medium, Nicoli and Vattier (1964) found the optimum inoculum of T.rhodesiense to be 100-1000 flagellates/mm^3, larger inocula resulting in rapid degeneration of the parasites. Even these two reports do not contain any evidence of detailed quantitative studies.

In the course of the present studies, the attempt at mass cultivation of T.brucei in the presence of G.morsitans tissues did not give rise to spectacular success. Under standard conditions, i.e. an inoculum of 1000 organisms/mm^3 and one complete alimentary tract, volumes of the medium exceeding 100 µl

were found less favourable for the cultivation of the trypanosomes. Smaller final populations were observed in such volumes than in those of less than 100 ul. It is known that in culture, insect organs and especially the alimentary tract, remain healthier when they are not covered completely by the medium, the most vigorous peristalsis occurring in organs which are only partially submerged. It seems probable that the poor growth of the trypanosomes suspended in larger volumes of the medium was caused by decreased viability of the insect tissues. In the standard hanging-drop preparations used throughout this study, the tsetse tissues were only partly covered by the medium containing the parasites. On the other hand, the poorer growth of the parasites with alimentary tract from pupae younger than 21 days was not caused by the smaller size of the explants which obviously affects the tissue volume: medium volume ratio. It was apparant that the stage of differentiation of the older alimentary tracts rather than their size affects the growth of the trypanosomes.

It is evident from the negative results obtained with the culture medium alone that in the system employed in the present study, the growth of the trypanosomes depends upon the presence of living Glossina tissues. The results of the experiments designed to elucidate the nature of the stimulatory affect of the tsetse alimentary tract upon the growth of the trypanosomes have shed some light upon this question. (1) Extracts of the gut failed to support multiplication of the parasites. Hence the chemical constituents of the explants alone were insufficient for stimulation and maintenance of the growth of the trypanosomes.

(2) Culture medium preconditioned by 3-4 days growth of tsetse tissue also was found incapable of inducing multiplication of the salivarian species. In view of this, one could question the importance of metabolic products of the insect alimentary tract either in stimulating growth of the flagellates or in counteracting the effects of possible inhibitory factors present in the in vitro system. It is likely, however, that the growth factors produced by the living tissues are too labile to exert their effect in the absence of the tissues. (3) That, indeed, certain substances of low molecular weight emanating from Glossina alimentary tract are essential for the growth of the trypanosomes was suggested by the results of experiments in which the parasites were separated by a semipermeable membrane from the insect tissues. It seems that studies conducted with the aid of this kind of system might provide an opportunity for the analysis of the nutritional requirements of the trypanosomes, leading ultimately to the development of more defined culture media for these parasites. Admittedly such an analysis probably would require larger volumes of the medium. This difficulty could be obviated by using alimentary tracts from several pupae on one side of the semipermeable membrane.

Haemin, most often provided in intact red cells or in the form of red cell lysates, has been thought to constitute an absolute requirement for growth of trypanosomatid genera and species in various culture media (Guttman and Wallace, 1964; Taylor and Baker, 1968). In media employed for the "lower" trypanosomatids (see Guttman and Wallace, 1964) and in the semi synthetic medium devised for the salivarian species (Cross and

Manning, 1973) purified haemin was employed, often in very low concentrations (for example, 10 mg/litre in Cross and Manning mixture). Haemin content of the foetal bovine serum incorporated in the medium used for the cultivation of tsetse tissues in the present studies varied between 70 and 100 mg/100ml. It was suggested (Fromentin, 1961) that HeLa and HEP cell cultures can support growth of Trypanosoma rhodesiense and T.gambiense because of the ability of these vertebrate cell lines to synthesize haemin. Whether the tsetse tissues also possess this capacity can be ascertained only by using a haemin-free medium. Development of such a medium is now in progress.

To reduce the time and effort required to maintain high level parasitaemias in laboratory rodents, the customary source of inocula for in vitro cultures, the use of trypanosomes stabilated in dry ice or liquid nitrogen with 7·5% of glycerol as the cryoprotectant was attempted. As detailed in the "Experimental Results", stabilates of T.brucei in mouse blood were found entirely satisfactory as inocula. Stabilates frozen in rat blood could not be used owing to the formation of crystals in infected frozen blood. These crystals persisted in cultures and presented difficulties in quantitative evaluation of the results. The lack of success with stabilates of Trypanosoma congolense was undoubtedly caused by the low recovery rate of viable organisms from thawed samples. It is possible that improved freezing procedures will produce stabilates of this latter species as suitable as those of T.brucei.

The field observations that species of Glossina differ in their ability to transmit trypanosomes (Lumsden, Cunningham, Harley, Southan, van Hoeve, Grainge, Wiggali, Ochieng and Highton,

1963; Willet, McMahon, Ashcroft and Baker, 1964) were subsequently confirmed by laboratory experiments (Harley and Wilson, 1968; Wilson, Dar and Paris, 1972). In the course of the present study, however, tissues derived from the two varieties of G.morsitans as well as from G.austeni and G.palpalis were found equally suitable for the cultivation of T.brucei. It must be emphasised, however, that unlike in the flies, in the presence of tsetse tissue cultures the development of the trypanosomes does not proceed beyond the midgut or at best to proventricular stages (Vickerman, personal communication). It is known also that especially with regard to T.brucei - subgroup species, percentages of flies with midgut infections markedly exceed those of vectors in which the development proceeds to "mature" infections involving the formation of infective stages in the salivary glands (Page, 1971; Dipeolu, 1972; personal unpublished observations). However, the workers who studied transmission of trypanosomes by different species of Glossina have paid only slight attention to midgut or proventricular infections, being more concerned with mature infections, the only ones important in transmission.

As it is not always possible to obtain regular supplies of tsetse pupae, it seemed of interest to investigate whether T.brucei could be cultivated in cultures of the alimentary tract from other more easily available haematophagous arthropods. Such cultures of tissues from developing adult ticks, Rhipicephalus appendiculatus, and adult mosquitoes, Aedes aegypti, grown in modified Trager's medium supplemented with homologous pupal extracts (for A.aegypti) as well as Schneider's (1969) and Varma

and Pudney's (1970) media did not support growth of the trypanosomes. The fact that the size of the alimentary tracts did not constitute a limiting factor was demonstrated by experiments in which three alimentary tracts from mosquitoes, which in their combined volume roughly corresponded to a single alimentary tract from Glossina, were grown in 15-20 µl of a culture medium, cultures of trypanosomes could not be established in this system. On the other hand, cultures of the complete alimentary tract from Sarcophaga, a non-haematophagous dipteran closely related to Glossina, were found to support the growth of trypanosomes. Unfortunately in many instances, the results with Sarcophaga tissues were vitiated by the presence in the gut of the flies of a trypanosomatid, tentatively identified as Herpetomonas muscarum (see Wallace and Clark, 1959). The presently available information does not justify any valid conclusions with regard to the aforementioned results. It is probable that other members of Cyclorrhapha, the suborder to which both Glossina and Sarcophaga belong, may provide suitable donors of tissues suitable for the in vitro cultivation of the salivarian trypanosomes. As far as can be ascertained from the literature, there is at present no information about the establishment of midgut or proventricular infections with these trypanosomes in cyclorrhaphan genera other than Glossina. From many viewpoints investigations along these lines would appear to be of both theoretical and practical interest.

After it was established in numerous experiments that organ cultures of the complete alimentary tract from Glossina provided a suitable environment for pleomorphic, fly-transmissible strains of T.brucei (TREU 667) and T.congolense (TREU 623 and 694),

cultivability of other salivarian strains and species in this system was investigated. Growth of pleomorphic T.rhodesiense (TREU 788) in the presence of Glossina tissues was closely comparable to that of T.brucei. Somewhat different results, however, were obtained with the predominantly monomorphic T.b.brucei Etat 5 strain derived from a clone isolated originally by McNeillage, Herbert and Lumsden, (1969). Unlike the pleomorphic strain of T.brucei, which in the presence of Glossina cultures entered the growth phase after a 24 hour lag, the initial inocula of Etat 5 trypanosomes exhibited a 2- or 3-day lag phase before exponential growth. Further, whereas the former strain reached its peak numbers in 5 days, those numbers were not attained by the latter until up to 10 days. These results indicated a longer generation time of Etat 5 in the initial cultures. Upon subculture, however, the fully transformed organisms of this strain assumed growth characteristics similar to those observed with the pleomorphic trypanosomes. No comparable differences were noted between a fly-transmitted (TREU 694) and laboratory-adapted (TREU 261) strain of T.congolense. Surprisingly, all attempts to establish in tsetse organ cultures Gray's (1972) L_2 and R_3 strains of T.gambiense resulted in failure. With these latter strains, the numbers of the parasites present in the original inocula decreased to about one-half and remained at these levels for 10 days when the experiment was terminated. It is possible that the apparent loss of cultivability by the two strains of T.gambiense was caused by the numerous passages in rodents to which they were subjected from the time of their isolation from human hosts to their employment in the experiments

detailed in this thesis.

A strain of T.evansi (TREU 381), a typically monomorphic species, could not be grown in tsetse organ cultures, the flagellates present in the original inocula died within 4 days. The "T.evansi-subgroup" parasites are known to be transmitted mechanically by Diptera other than Glossina. When ingested by tsetse flies, these trypanosomes die within 6 hours and are digested together with the blood meal (Hoare, 1940). All attempts to cultivate the "T.evansi-subgroup" strains in vitro resulted in failure, although their survival for up to 20 hours in liquid medium containing certain amino acids was reported by Balis (1963, 1964a, b). It seems that like the non-living media, the tsetse culture system employed in this present study is incapable of supporting growth of salivarian trypanosomes which do not undergo cyclical development in the insect vector. These results are not surprising in the light of the information available on the fine-structural and biochemical aspects of transformation of the tsetse-transmitted pleomorphic strains (Vickerman, 1965, 1969, 1971; and see Honigberg, 1967 for review of the biochemical aspects). It is evident from the data available at present that the exclusively monomorphic T.evansi is no longer capable of transforming into stages equipped with a functional chrondriome and thus lack the potential for changing their energy metabolism to one involving the enzymes of the tricarboxylic acid cycle and cytochromes. Since the mitochondria-linked pathways are known to be utilized by all the procyclic trypanosomes, the inability of "T.evansi-subgroup" parasites to develop in Glossina or to grow in culture is to be

expected.

The growth pattern of the pleomorphic strain of T.brucei in tsetse organ cultures is closely comparable to that reported for similar strains of T.rhodesiense and T.gambiense in the presence of mammalian cell lines (Demarchi and Nicoli, 1960) and for pleomorphic T.brucei in Pittam's medium (Brown, Evans and Vickerman, 1973). According to Brown et al. (1973) inocula of T.brucei containing predominantly long slender bloodstream forms failed to initiate cultures in Tobie's medium, or at best little growth resulted from such inocula. On the other hand T.brucei trypanosomes derived from irradiated rats, in which the parasite population contained numerous short stumpy stages, gave rise to fast growing cultures in this medium. The results reported by Cross and Manning (1973) appear to be at variance with those of Brown et al. (1973) and with the observations detailed in this present thesis. Using their modification of Pittam's medium, Cross and Manning found that it took 30 days to establish a culture of a fly-transmissible T.brucei strain, but that a monomorphic strain of this species gave rise to a rich culture within 15 days. These observations are difficult to reconcile with the current information regarding the physiological aspects of the blood stream-to-culture form transformation (Vickerman, 1971, 1972; Evans and Brown, 1972; Brown et al., 1973 and see Honigberg, 1967 for review of the earlier reports). According to all experimentally derived data it appears that it is the stumpy forms of the pleomorphic species and strains of the subgenus Trypanozoon that are capable of transformation. The monomorphic strains of all Trypanozoon species and the exclusively monomorphic species of

the "T.evansi-subgroup" of this subgenus have not been found capable of establishing themselves either in cultures or in tsetse flies. It has been suggested by Vickerman (1965) that the short stumpy forms alone are preadapted to acquiring the structural and biochemical attributes requisite for survival in the vector's gut and/or culture. The arguments put forward by Omerod and Vankatesan (1974) that the short stumpy forms actually are degenerate stages not actively involved in the life cycle of Trypanozoon spp. are not supported by adequate evidence; they could not be adduced in explanation of the results obtained by Cross and Manning.

The failure to cultivate T.vivax in the tsetse organ culture system deserves special attention. Inocula of the two strains of this species, the rodent-adapted TREU 65 of Desowitz and Watson (1951) and the fly-transmissible TREU 1011, degenerated quite rapidly, no living organisms remaining by the 4th day. No improvement with regard to the rodent-adapted strain was achieved by substituting mouse serum for the foetal bovine serum in the medium. Trypanosoma vivax is a monomorphic species and its life cycle does not include any development in the midgut or proventriculus of Glossina. In general, monomorphic strains of even pleomorphic species are not cultivable and the forms invariably observed in all known types of culture are the midgut- and proventricular-like stages. These facts may explain the lack of success in cultivating T.vivax in the system employed in the present study. On the other hand, Vickerman (1965) reported well developed cristae in the chrondriome in the bloodstream forms of this species in which a positive cytochemical

formazan reaction for diaphorase was also observed. Admittedly, however, the situation with regard to the energy metabolism pathways in T.vivax is by no means clear. Indeed, it is not certain how much energy may be derived from the cytochrome-linked reactions (see Honigberg, 1967 for pertinent references). By far the most surprising findings are the differences between the present results and those of Trager (1959a, b) who was able to grow several strains of T.vivax in cultures of the salivary glands and alimentary tract from G.palpalis maintained at 28-31°C. One of the differences between his system and that described in this present investigation was the species of the flies. Moreover, Trager's strains were different from those used here, and according to him, certain isolates from infected sheep were unsuitable for cultivation. It can not be deduced that the use of homologous serum by Trager was responsible for his success, as, in the cultures employed in the present studies, growth was not improved by incorporating calf serum into medium to be used for the cultivation of a strain derived from calf's blood. It should be pointed out, however, that the stabilates used in the experiments might have been unsuitable for initiation of cultures. Further experiments with trypanosomes derived directly from the blood of infected mammalian hosts as well as with tissues from different species of Glossina may ultimately bring about the cultivation of T.vivax in the system found suitable for the other salivarian species. Until success is achieved in growing T.vivax at 28-31°C, no meaningful attempts can be made to produce infective stages of this trypanosome by incubating the cultures at 37°C, the procedure used by Trager

in obtaining such forms.

It was reported recently that flagellates morphologically resembling metacyclic trypanosomes were present in tsetse haemolymph (Mshelbwala, 1972). In view of this finding and also in an attempt to develop a culture medium more chemically defined than those now available, amino acid analyses of G.morsitans haemolymph were undertaken in collaboration with Dr. J.S. Slater (Cunningham and Slater, 1974). Certain differences in amino acid composition and in the concentration of individual amino acids were noted among haemolymph samples taken from 10 day pupae, pre-emerged flies and 2-day adults. Although such differences could have been anticipated on the basis of previously published reports dealing with metabolism of other insect species (Florkin and Jeuniaux, 1964, Chen, 1966), their more precise interpretation would require more knowledge of the various facets of tsetse fly metabolism than is available at present. A certain amount of speculation seems justified. Of special interest was the finding of the high proline concentration, particularly in the pre-emerged flies (1088 mg/ 100 ml). A high level of this amino acid was reported previously from homogenates of G.palpalis (Balogun, 1969, 1971). It is known that proline participates in insect protein metabolism, being a major component of the cuticle (Hackman, 1953). Further an analysis of G.morsitans musculature during the hunger cycle revealed the presence of high levels of proline in resting flies and their decrease during flight (Bursell, 1963). In view of the foregoing findings, it would seem likely that the reduced proline concentration in the 2-day flies was related to cuticle

formation or flight, or to both of these physiological functions. The large increment in the alanine concentration in 2-day flies confirmed the findings of Bursell (1963), according to which the level of this amino acid increased dramatically after flight. High concentrations of alanine were demonstrated also by paper chromotography of haemolymph from G.pallidipes (Knight, 1961) and extracts of whole G.palpalis (Balogun, 1971). The fluctuation in tyrosine concentration observed in the present analysis could be explained by the general accumulation of tyrosine in the blood during adult development in preparation for the final stages of sclerotization and tanning of the cuticle which takes place at emergence of adult flies. It has been ascertained that in general, tyrosine plays an important role in the metabolic activities involved in insect metamorphosis (Bursell, 1970).

The data on the amino acid composition of tsetse haemolymph obtained in the course of these studies have been used as a basis for the formulation of media with a more defined chemical composition for the cultivation of tsetse tissues and trypanosomes than has been hitherto possible.

The first medium devised on the above basis was found capable of supporting multiplication of T.brucei (TREU 667), but only in the presence of living tsetse alimentary tract. In the primary cultures as well as upon transfers, the growth characteristics of this species in the new medium were very similar to those which it exhibited in the modified Trager's medium. Different results were obtained, however, with T.congolense (TREU 694). Although conditions similar to those obtaining in the latter medium were necessary to establish these parasites in primary

cultures, upon subculture, the tsetse organ cultures were no longer required and the trypanosomes grew well in much larger volumes of the new medium (0·4 ml vs 20 µl). Cross and Manning (1973) were able to grow T.brucei and T.rhodesiense in semi-defined media, but similarly to T.congolense in the amino acid medium, primary cultures of the "T.brucei-subgroup" trypanosomes could not be initiated in their media with inocula derived directly from infected animals; only flagellates already transformed and established in Pittam's medium were capable of multiplying in the semi-defined mixtures. Of special interest with regard to cultivation of T.congolense in the amino acid No. 2 medium was the development of stages which in their morphology resembled the infective forms of this species. These forms developed at pH 7·4. Since it is known that the pH of tsetse saliva ranges from 7·4 to 9·0 (Lester and Lloyd, 1928; Williamson, 1956; Dar, 1971) hydrogen ion concentration falling within this range would be expected to favour the formation of the metacyclic like stages. Among other factors, osmolarity of the medium might have been effective in stimulating the formation of such stages. It was ascertained experimentally that the osmolarity of amino acid No. 2 medium was higher (350 m Osmoles/kg) than that of the modified Trager's medium without pupal extract (253 m Osmoles/kg). Although the effect of osmolarity upon the development of trypanosomes has not been explored to date, the apparent preference of culture forms for media with high osmotic pressure may be related to the reportedly hypertonic conditions prevailing in the tsetse midgut (Bursell, 1970). At this time it is difficult to discuss the factors

affecting the differences between the strains of the two species. Some modifications in the composition and in pH may lead to the development of media that would be equally suitable for the cultivation of both species.

Invariably the establishment of trypanosome cultures in the presence of Glossina organ cultures was preceded by a morphological transformation of the bloodstream forms into stages morphologically similar to those found in tsetse midgut and proventriculus, and in cultures grown in mono- or diaphasic non living media (for review see Tobie, 1964; Bishop, 1967; Zeledon, 1971). Electron-microscopic investigations of the bloodstream-to-culture form transformations have provided ample evidence that the morphological changes observed with the aid of the light microscope reflect large alterations at the fine-structural level, especially with regard to the mitochondrial tubes and cristae mitochondriales, which are far better developed in the culture stages (for review see Vickerman, 1971, 1972). All the structural changes are known to underline modifications in the metabolic pathways (for review, see Honigberg, 1967; Vickerman, 1971, 1972; Newton, Cross and Baker, 1973). It has been assumed without conclusive proof, that the culture forms, the morphology of which closely resemble those found in the tsetse gut (Thomson and Sinton, 1912), are physiological equivalents of those in the vectors. Although such an assumption may be employed in a working hypothesis, further experimental work is required in this field.

The transformation of the pleomorphic T.brucei and T.congolense strains in the system used during the present study conformed to the generally accepted assumption that the short stumpy forms are the

ones capable of structural and functional transformation in cultures and in the flies. On the other hand, the establishment of cultures of the primarily monomorphic Etat 5 strain of T.brucei was quite unexpected. Presumably the long slender forms that constitute the population of monomorphic strains are non infective to flies and cannot be used for initiation of cultures. A similar difficulty in interpretation exists with regard to the alleged monomorphic strain of T.brucei cultivated by Cross and Manning (1973). The strain of T.brucei (Etat 5) was found non-infective to tsetse flies (Page, personal communication) but no comparable information is available about the strain used by Cross and Manning. On the other hand, no attempts have been made so far to cultivate Etat 5 in non-living media, for example Tobie's or Pittam's, generally used for growing salivarian trypanosomes in vitro. Much further research will have to be done on various aspects of the problem before the cultivation of monomorphic strains can be explained in rational terms.

A great deal of evidence has accumulated during the last 50 years in support of the view expressed by some of the earlier investigators that transformation of the salivarian trypanosomes in the gut of tsetse flies or in culture is accompanied by a loss of infectivity by these parasites (for pertinent references see Mendez and Honigberg, 1972). It was shown in the present study that a pleomorphic strain of T.brucei (TREU 667) retained its infectivity to mice after 4 days of cultivation in the presence of tsetse organ cultures. A similar strain of T.congolense, however, lost its infectivity to mice after 24 hours in culture. With T.brucei, the prepatent period varied from 5 days for 1-day

cultures to 8 days for cultures maintained for 4 days. It was shown recently that infectivity to mice of cultures of several T.brucei and T.rhodesiense strains depended upon the persistence of non-transformed bloodstream stages; when these stages disappeared, the cultures ceased to be infective (Mendez and Honigberg, 1972). The periods between initiation of cultures and loss of infectivity varied from strain to strain. Observations made in the course of the investigations described in the present thesis indicated that by the 4th day of cultivation the T.brucei populations were in the late stage of their logarithmic growth and no longer contained bloodstream forms. Despite careful examination of stained preparations, the possibility that a few of these forms were present cannot, however, be eliminated. It was demonstrated by electron microscopy that bloodstream forms of the subgenera Trypanozoon, Duttonella and Nannomonas are invested with a surface coat. This coat, lost during transformation of the bloodstream trypomastigote into the procyclic forms, is reformed during transformation of the epimastigote to the metacyclic stages, irrespective of whether this latter process occurs in the salivary glands (Trypanozoon) or proboscis (Duttonella and Nannomonas) (Vickerman, 1969; Steiger, 1971). It has been suggested that the coat provides the variant agglutinogens (see Vickerman, 1969, 1971, 1972; Vickerman and Luckins, 1969). According to the findings of Cunningham and Latif (1973) and Latif (1973), however, antiserum to bloodstream forms was capable of agglutinating T.brucei cultured for 3 days at titres equal to those observed with their bloodstream forms homologous to the particular immune serum. Although at a

slightly lower titre this antiserum also caused agglutination of flagellates maintained for 4 days. Similar results were obtained by the indirect fluorescent antibody test (IFA) (Latif and Adam, 1973), the titre decreasing in preparations containing trypanosomes from 4 day cultures. Electron microscopic examination of T.brucei (TREU 667) from 3 day cultures grown in the presence of Glossina tissues indicated that the flagellates lacked the surface coat and their mitochondria resembled those typical of the proventricular forms (Vickerman, personal communication). In the light of these observations the infectivity and agglutinability of the cultured trypanosomes are difficult to explain in the framework of present knowledge. Perhaps the persistance of a few bloodstream forms in the cultures was responsible for the retention of infectivity. It is also possible that the agglutinability of the cultured trypanosomes was due to the presence of the surface coat protein which might have been dissolved in the small drops of culture medium from which the flagellates were transferred to the drops of immune serum. Further immunological studies combined with fine-structural examinations are required to explain the results obtained with the cultures. One of the first steps would involve washing the trypanosomes from the culture medium to eliminate the possibility of the presence of dissolved agglutinogens which may have come from the surface coat of the no longer surviving bloodstream forms.

There have been sporadic reports of the reappearance of infectivity to laboratory rodents in cultures of Trypanozoon spp. maintained in various non-living media (Weinman, 1957, 1960;

Geigy and Kauffman, 1964; Amrein, Geigy and Kauffman, 1965; Cross and Manning, 1973; Amrein and Hanneman, 1969, 1974). Unfortunately data on the fine structure of the infective stages is not available. As pointed out by Mendez and Honigberg (1972) these results are difficult to explain in the light of the present information on the factors controlling infectivity of the cyclically transmitted salivarian trypanosomes.

So far Trager (1959a, b) alone has restored infectivity of T.vivax cultivated in the presence of Glossina organ cultures to sheep. According to him, a 19-hour exposure to 37°C of actively growing parasite cultures was responsible for this restoration of infectivity. A similar treatment of T.brucei and T.congolense cultures obtained in the present study failed to restore infectivity to these trypanosomes. On some occasions metacyclic-like trypomastigotes were observed, especially in the cultures of T.congolense grown in the amino acid medium No. 2. Such trypanosomes inoculated into mice via the subcutaneous and intradermal route to simulate inoculation by tsetse flies, never produced parasitaemias in the experimental animals. As pointed out elsewhere in this discussion, the differences between the results of Trager and those reported in this thesis might have depended upon several factors.

The use of tsetse organ cultures improved in vitro growth of several species of the stercorarian trypanosomes and appeared to extend the period of their infectivity to laboratory animals. For example both T.musculi and T.lewisi exhibited more luxuriant growth when cultured in this system than in the modified Trager's

medium alone. The former species remained infective to mice for 28 days in cultures containing living tsetse organs but for only 8 days in those with the medium alone. Comparable, although not as good results were obtained when T.musculi was cultivated with alimentary tract from developing adult Rhipicephalus appendiculatus in VP_4 medium. Although metacyclic forms were noted in cultures of T.musculi and T.lewisi, the most interesting results relating to such forms were obtained with similar cultures of T.melophagium and T.theileri. When initiated either from epimastigotes grown on blood agar slants or directly from stabilates, cultures of both these species grew better in the presence of tsetse alimentary tract than in the medium alone. On about the 10th day, the long epimastigotes changed into shorter forms which morphologically resembled those found in the hindgut of the vectors. In cultures of T.theileri the shorter forms included the "barley corn" organisms believed to be the infective metacyclic stages (Noller, 1925). Unfortunately infectivity of either T.theileri or T.melophagium to their natural mammalian hosts could not be tested. This aspect of research could constitute a most interesting subject for future investigations.

It is evident from the results of the studies detailed in this thesis that they posed more questions than they answered. However, at least some of the observations might prove useful in that they have provided means whereby several important problems of cultivation, immunology and infectivity factors can be attacked in a manner potentially more promising than has hitherto been possible.

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APPENDIX

Sources of Chemicals; Reagents and Biological Stains

Decon 75 liquid detergent	from	Decon Laboratories Ltd., Brighton.
Roccal antiseptic containing 1% benzalkonium cloride	from	Pfizer Ltd., Sandwich, Kent.
The salts, organic acids, sugars, amino acids, were Analar quality	from	B.D.H. Ltd.
Eagles basal medium and Vitamin B mixture (100x conc.)	from	Wellcome Reagents Ltd.
Grace's medium prepared by Grand Island Biological Co.	from	Tissue Culture Services Ltd., Slough.
Foetal bovine serum	from	Flow Laboratories, Irvine, Ayrshire.
Bovine albumin Powder (Fraction V)	from	Sigma, London.
'Marmite' - yeast extract prepared by		Marmite Ltd., Enfield, Middsex.
Yeast extract, Lactalbumin, T.C. Yeastolate and Trypsin 1:250	from	Difco Laboratories.
Fungizone (Amphotericin B) for Tissue Culture, E.R. Squibb & Sons Inc. product.	from	Vestric Ltd., Edinburgh.
Streptomycin Sulphate B.P. and Crystopen (Benzylpenicillin B.P.) prepared by Glaxo Laboratories	from	Vestric Ltd., Edinburgh.
Heparin Injection B.P. 500 units per ml.	from	Boots Co. Ltd.
All biological stains and mounting media for permanent preparations were obtained from		George T. Gurr, High Wycombe, Bucks.

White's solution

0·25	gm	HgCl
6·5	gm	NaCl
1·25	gm	HCl
250	ml	ethanol
750	ml	distilled water

Lactic acetic orcein stain

1 gm or natural orcein is dissolved in a solution consisting of 50 ml 70% lactic acid and 50 ml glacial acetic acid previously heated to 80°C. The stain is allowed to cool then filtered, left overnight and refiltered before use.

Insect balanced salt solution (IBSS) consists of two solutions prepared as follows:

Solution A	7·5	gms	NaCl
	0·2	gms	KCl
	0·2	gms	$CaCl_2$
	2·0	gms	Glucose
	0·02	gms	Phenol red

dissolved in 900 ml distilled water and autoclaved at 10 lbs/sq. in. for 15 minutes.

Solution B	0·2	gms	$NaHCO_3$ dissolved in 100 mls and filtered by Millipore filtration.

Modified Trager's Medium (Medium No. 9)

Solution A	mgm/100 ml
NaCl	90
KCl	300
$NaH_2PO_4.2H_2O$	110
$MgSO_4.7H_2O$	370
$CaCl_2.2H_2O$	80
Glucose	150
Trehalose	50
L-Malic acid	50
α-Ketoglutaric acid	25
Succinic acid	5
Lactalbumin hydrolysate (Difco)	1,000
Yeast extract (Difco)	200

Ingredients dissolved in distilled water and the pH adjusted to 6·7 with 1·0N NaOH.

Solution B	mgm/10 ml
Reduced Glutathione	200
Ascorbic acid	2

Both solutions sterilized by filtration.

The dissecting solution (DS) was composed of 8·0 ml of solution A, 0·5 ml of solution B and 2·0 ml foetal bovine serum. Penicillin and streptomycin were added to give a final concentration of 100 IU/ml.

To prepare the culture medium, the dissecting solution was enriched with tsetse pupal extract. Four or five surface sterilized pupae aged 8-12 days were crushed in 1·0 ml of DS. The mixture was centrifuged at 2,200g for 10 minutes and the clear supernatant fluid was decanted off as the culture medium.

Osmolarity of DS = 253 m Osmoles/kg.

Varma/Pudney Medium 4 (VP_4) for tick tissues

NaCl	390 mgs
$NaH_2PO_4.2H_2O$	55 mgs
$MgCl_2$	110 mgs
$MgSO_4$	120 mgs
$CaCl_2.2H2O$	40 mgs
KCl	55 mgs
Glucose	200 mgs
Inositol	40 mgs
Lactalbumin hydrolysate	500 mgs
$NaHCO_3$	50 mgs
B.P. Albumin	50 mgs
Glutamine 5%	0·6 ml
Fagles Vitamin Mixture	1·0 mls (10x conc.)
Distilled water	98·4 mls

Adjust pH to 6·9 with 2% KOH. Sterilize by Millipore filtration and add 10% foetal bovine serum to make up the complete medium. Penicillin and streptomycin were added to give a final concentration of 100 IU/ml.

Osmolarity 245 m Osmoles/kg.

Eagles-Vago-Chastang (E-V-C) Medium for tick cells

$NaH_2PO_4H_2O$	120 mgs
$MgCl_26H_2O$	300 mgs
$MgSO_47.H_2O$	400 mgs
KCl	300 mgs
$CaCl_22H_2O$	100 mgs
Trehalose	150 mgs
Loctalbumin hydrolysate	1,100 mgs
Phenol red - 0·2%	1 ml
Distilled water	99 ml

pH adjusted to 6·4 with 5% KOH. Sterilize by filtration and add 10% foetal bovine serum. Penicillin and streptomycin added to give a final concentration 100 IU/ml.

Amino acid medium No. 1 based on Trager's Medium and the composition of amino acids in tsetse pupal haemolymph

All ingredients in mgm/100 ml

NaCl	90	Methionine	12
KCl	300	Threonine	45
$NaH_2PO_4.2H_2O$	110	Serine	60
$MgSO_4.7H_2O$	370	Glutamine	126
$CaCl_2$	80	Asparagine	57
Glucose	150	Glutamic acid	203
Trehalose	50	Proline	239
L-Malic acid	50	Glycine	59
∝ Ketoglutaric acid	25	L-Alanine	142
Succinic acid	5	Valine	86
		iso-Leucine	36
Eagles Vitamin B Mixture (10x conc.)	1·0 ml	Leucine	40
		Tyrosine	50
		Arginine	201
		Aspartic acid	10
		Cystine	2·5
		Cysteine	5·0
		Tryptophan	10

Adjust pH to 6·7 with 1·0N NaOH, sterilize by filtration and add 20 ml foetal bovine serum. Penicillin and streptomycin were added to give a final concentration of 100 IU/ml.

Amino acid medium No. 2 based on salts, sugars, organic acids of Grace (1962) and the concentration of amino acids which occurred in haemolymph of 2 days old tsetse flies. All ingredients in mg/100 ml.

Inorganic Salts		Amino Acids	
NaH_2PO_4	110	Threonine	10
$MgCl_26H_2O$	304	Serine	20
$MgSO_47H_2O$	370	Glutamine	164
KCl	298	Asparagine	24
$CaCl_2$	81	Glutamic acid	25
		Proline	690
Sugars		Glycine	12
Glucose	70	β-Alanine	200
Fructose	40	DL-Alanine	109
Sucrose	40	Valine	21
		iso-Leucine	9

Organic Acids	
Malic	67
Ketoglutaric	37
Fumaric	5·5
Succinic	6

Taurine	27
Leucine	9
Tyrosine	20
Phenylalanine	20
Lysine	15
Histidine	16
Arginine	44
Aspartic acid	11
Tryptophan	10
Cystine	3
Cysteine HCl	8
Methionine	20

Preparation of the Medium

Dissolve Inorganic salts (except $CaCl_2$) in 10 mls distilled water.

$CaCl_2$	" 5 mls	"	"
Sugars	" 10 mls	"	"
Organic acids	" 5 mls	"	"
Amino Acids	" 65 mls	"	"
Vitamin B mix (100x conc.)	" 0·1 ml	"	"

Adjust pH to 7·4 with 10% KOH. Sterilize by Millipore filtration and add 20% foetal bovine serum. Penicillin and streptomycin were added to give a final concentration of 100 IU/ml.

Osmolarity = 350m Osmoles/kg.

Appendix table 1

Growth of cells derived from trypsinized developing adult tissues from Rhipicephalus appendiculatus. Means and standard deviations of three cultures in each series.

Days	Primary cultures Mean ± SD		1st Passage Mean ± SD		2nd Passage Mean ± SD	
5hr	7·1	0·6	7·5	2·1	7·8	0·7
2						
3	7·0	1·4				
4			8·5	2·8	9·6	1·7
5	10·3	1·9				
6						
7	12·7	1·7				
8			12·6	4·5	10·5	1·6
9	16·9	2·5				
10						
11	19·7	3·1				
12			18·2	3·3	13·6	2·0
13	23·7	0·6				
14						
15	24·5	0·5				
16			21·3	3·8	15·9	2·3
17	24·9	0·1				
18						
19						
20			23·1	2·4	16·5	1·3
21						
22						
23						
24			24·1	1·6	19·8	1·3
25						
26						
27						
28			24·7	0·2	21·6	1·9
32					23·8	0·8

Appendix table 2

Growth of *Trypanosoma brucei* in cultures of alimentary tract from 22-27 day old pupae, *Glossina morsitans*, and in culture medium alone. Means and standard deviations of four experiments.

	Means and standard deviations (SD) of No. of trypanosomes/mm^3			
	With alimentary tract		Medium alone	
Days	Mean	± SD	Mean	± SD
0	910	45	910	45
1	850	63	522	62
2	2,537	820	281	49
3	8,234	1,348	172	62
4	17,918	5,037	54	39
5	24,041	3,400	12	3
6	28,150	1,233	0	0
7	25,615	3,576		
8	17,197	4,804		
9	5,592	2,400		
10	3,065	1,492		
11	802	491		
12	935	686		
13	745	446		
14	117	164		

Appendix table 3

The effect of the inoculum size of suspensions on the growth of Trypanosoma brucei (TREU 667) in cultures with Glossina morsitans pupal alimentary tract. Means and standard deviations of four experiments.

Days	Means and standard deviations (SD) of No. of trypanosomes/mm^3							
	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD
0	142	50	389	36	662	17	1,077	38
2	127	41	687	121	4,459	980	6,872	1,268
4	173	43	11,555	1,064	18,878	1,862	26,862	1,696
6	321	48	15,185	585	24,857	1,737	32,732	858
8	316	136	16,992	1,850	29,085	2,732	28,265	3,456

Appendix table 4

The effect of the inoculum size of suspensions on the growth of Trypanosoma congolense (TREU 694) in cultures with alimentary tract from Glossina morsitans pupae. Means and standard deviations of four experiments.

Days	Means and standard deviations (SD) of No. of trypanosomes/mm^3							
	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD
0	95	14	326	48	643	94	1,052	67
2	199	53	18,037	394	4,391	831	6,879	1,267
4	994	110	11,530	778	20,148	1,112	27,219	1,186
6	5,842	196	23,308	914	27,346	732	32,001	861
8	15,820	1,877	28,329	926	28,796	1,280	26,061	1,241

Appendix table 5

Growth of Trypanosoma brucei (TREU 667) in different volumes of culture medium containing alimentary tract from Glossina morsitans pupae aged 22-27 days. Means and standard deviations of four experiments.

	Means and standard deviations (SD) of No. of trypanosomes/mm^3							
	20 µl medium		100 µl medium		0·5 ml medium		1·0 ml medium	
Days	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD
0	987	88	987	88	987	88	987	88
1	868	35	857	39	822	32	782	22
2	3,915	371	1,542	400	950	69	905	1,045
3	12,450	743	3,711	410	1,617	436	1,943	149
4	26,900	611	8,872	1,100	2,941	292	4,266	1,062
5	32,629	2,208	12,473	1,361	5,451	1,130	5,537	974

Appendix table 6

Growth of Trypanosoma brucei (TREU 667) when serially passaged every fifth day in the presence of tsetse pupal alimentary tract, and in culture medium alone. Means and standard deviations of four experiments.

Days	Mean and standard deviation (SD) of No. of trypanosomes/mm^3					
	With Alimentary Tract			Without Alimentary tract		
	Mean	±	SD	Mean	±	SD
0	962			962		
1	848		71	552		63
2	1,991		1,116	371		114
3	6,996		2,262	148		32
4	15,971		5,054	62		27
5	20,293		3,269	0		
5*	940			940		
6	2,207		1,682	434		358
7	10,665		1,019	271		84
8	15,861		520	163		82
9	16,522		3,094	101		22
10	21,447		2,531	7		12
10*	1,103			1,103		
11	3,381		1,470	738		162
12	6,661		733	489		56
13	15,992		970	171		60
14	25,937		1,965	30		40
15	30,044		1,303	0		
15*	865			865		
16	873		114	622		40
17	3,410		815	362		102
18	13,388		891	199		46
19	21,664		1,353	44		51
20	26,761		814	0		
20*	887			887		
21	899		242	481		79
22	3,879		728	160		39
23	8,520		769	107		49
24	18,161		1,745	43		33
25	26,726		1,012	0		
25*	1,100			1,100		
26	1,075		226	730		186
27	4,036		656	570		60
28	7,027		1,280	332		88
29	9,097		1,256	106		48
30	13,147		2,354	12		13

* The trypanosomes were subcultured every fifth day.

Appendix table 7

Growth of Trypanosoma brucei (TREU 667) in cultures of different tissues of pupae aged 25-27 days and in culture medium alone. Means and standard deviations of six experiments.

	Means and standard deviations (SD) of No. of trypanosomes/mm^3							
	Alimentary tract		Brain and Salivary glands		Body wall		Medium alone	
Days	Mean	± SD	Mean	± SD	Mean	± SD	Mean	± SD
0	1,148	46	1,148	46	1,148	46	1,148	46
1	955	78	889	56	770	52	700	60
2	4,523	1,190	2,636	989	1,085	138	366	73
3	11,025	1,008	6,928	1,425	1,845	594	154	57
4	22,960	4,015	12,317	914	3,857	821	38	25
5	30,075	1,434	17,127	1,915	6,086	597	5	9

Appendix table 8

The growth of Trypanosoma brucei (TREU 667) in cultures of tsetse pupal alimentary tract of different ages. Means and standard deviations of six experiments.

Days	Medium alone Mean ± SD		7 days Mean ± SD		14 days Mean ± SD		21 days Mean ± SD		27 days Mean ± SD		Adult flies Mean ± SD	
0	962	86	962	86	962	86	962	86	962	86	962	86
1	516	146	745	130	838	109	876	85	847	81	857	69
2	177	45	642	182	1,060	224	2,968	97	3,310	557	2,660	493
3	60	27	983	282	3,891	458	7,046	892	9,165	484	8,669	816
4	7	7	2,137	1,163	7,409	876	15,955	4,323	23,901	1,603	22,669	3,240
5	0·8	2	3,900	2,145	13,362	796	22,048	3,229	29,150	1,423	29,036	1,967

Appendix table 9

Growth of Trypanosoma brucei (TREU 667) in direct contact with pupal alimentary tract, in slide tissue culture chambers and in the absence of tsetse tissues. Means and standard deviations of four experiments.

	Means and standard deviations (SD) of No. of trypanosomes/mm^3					
	Alimentary tract		Culture chambers		Medium alone	
Days	Mean	± SD	Mean	± SD	Mean	± SD
0	1,015	85	1,015	85	1,015	85
1	887	107	742	103	647	76
2	1,287	361	802	215	323	59
3	7,941	3,942	4,253	1,172	149	57
4	22,171	4,741	14,215	3,206	43	23
5	28,592	3,324	18,380	2,715	2	2

Appendix table 10

Growth of Trypanosoma brucei (Etat 5) in cultures of alimentary tract from 25-27-day-old tsetse pupae and in culture medium alone. Means and standard deviations of four experiments.

Days	Means and standard deviations (SD) of No. of trypanosomes/mm^3 with Alimentary tract Mean	± SD	Medium alone Mean	± SD
0	806	79	806	79
2	672	83	281	32
4	2,835	1,152	53	21
6	12,524	1,664	0	0
8	27,937	1,707		
10	33,536	2,297		
10*	895	49	895	49
11	925	199	650	76
12	4,960	621	210	38
13	12,680	2,155	70	24
14	20,153	1,408	8	10
15	23,443	3,280	0	0

* Cultures were subcultured on the 10th day.

Appendix table 11

Growth of Trypanosoma theileri (TREU 641) in cultures of alimentary tract from 25-27 day old pupae, G.morsitans and in culture medium alone. Means and standard deviations of six experiments.

	Mean and standard deviation (SD) of No. of trypanosomes/mm^3					
	With Alimentary tract		Medium alone			
Days	Mean	± SD	Mean	± SD	t	P
0	887	45	887	45		
1	1,615	465	935	94	3·506	< 0·001
2	23,850	670	12,985	769	25·588	< 0·001
3	33,724	1,174	15,970	782	30·824	< 0·001
3*	833	159	833	159		
4	7,282	1,192	3,374	726	6·856	< 0·001
5	19,901	1,219	13,537	842	10·530	< 0·001
6	31,861	1,180	19,699	644	21·985	< 0·001
6*	802	38	802	38		
7	8,450	905	3,914	504	10·723	< 0·001
8	17,497	723	8,912	796	19·546	< 0·001
9	24,547	763	14,042	1,254	17·522	< 0·001

* the trypanosomes were subcultured every third day.

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Quantitative Studies on Trypanosomes in Tsetse Tissue Culture

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CUNNINGHAM, ISABEL. 1972. Quantitative studies on trypanosomes in tsetse tissue culture. *Experimental Parasitology* 33, 34–45. Tissues from pupae of *Glossina morsitans* of various ages were cultured in modified Trager's medium. Cellular outgrowths were produced from explants of proventriculus, brain, and imaginal body wall and large vesicles were extruded from pieces of midgut of young pupae. Complete alimentary tract from older pupae displayed rhythmic contractions for up to 3 weeks. When *Trypanosoma brucei* and *T. congolense* in mouse blood were added to hanging drop cultures of tsetse tissues and incubated at 28 C, the organisms multiplied and changed into forms morphologically similar to those found in the tsetse fly midgut. The trypanosomes were maintained for 30 days by serial passage at 5-day intervals. The growth of *T. brucei* in the presence of different pupal tissues was studied. Of all the tissues tested the complete alimentary tract from pupae older than 21 days gave the best results. Growth also occurred when the trypanosomes were separated from the insect tissue by a semipermeable membrane. The trypanosomes failed to grow in (a) culture medium alone, (b) media containing extracts of alimentary canal and (c) medium in which alimentary tract had been cultured for 3 or 4 days.

INDEX DESCRIPTORS: *Trypanosoma brucei; Trypanosoma congolense;* Tissue culture; *Glossina morsitans;* Life cycles.

The cultivation of mammalian trypanosomes dates from the beginning of this century when Novy and MacNeal described established cultures of *Trypanosoma lewisi* (1903) and *T. brucei* (1904) in "condensation water" which collected at the base of a mixture of nutrient agar and blood solidified in test tubes. Many blood-enriched culture media have been designed for the growth of trypanosomes and recent advances in this field have been reviewed by Tobie (1964), Trager and Krassner (1967), and Taylor and Baker (1968).

Trager (1959) introduced bloodstream forms of *T. brucei, T. congolense* and *T. vivax* into cultures containing surviving alimentary tract and salivary gland tissues of the tsetse fly *Glossina palpalis.* He observed rapid growth and transformation of the parasites and found that *T. vivax* developed to the infective metacyclic stage. Five years later Nicoli and Vattier (1964) grew *T. rhodesiense* in tissue cultures of *G. fuscipes.*

None of these workers, however, made any attempt to quantify the growth of the trypanosomes in their cultures. This report describes methods for the cultivation of tissues of the tsetse fly *G. morsitans* and experiments on the growth of *T. brucei* and *T. congolense* in these cultures.

MATERIALS AND METHODS

Solutions

The culture medium and solution for dissections were based on those used by

Trager (1959) but the sheep serum was omitted. Solution A was prepared by dissolving the following in 100 ml distilled water: NaCl, 90 mg; KCl, 300 mg; $NaH_2PO_4/2H_2O$, 110 mg; $MgSO_4/7H_2O$, 370 mg; $CaCl_2$, 80 mg; glucose, 150 mg; trehalose, 50 mg; L-malic acid, 50 mg; α-ketoglutaric acid, 25 mg; succinic acid, 5 mg; lactalbumin hydrolysate (Difco), 1000 mg; yeast extract (Difco), 200 mg. The pH was adjusted to 6.7 with 1 *N* NaOH. Solution B contained 200 mg reduced glutathione and 2 mg ascorbic acid in 10 ml distilled water. Both solutions were sterilized by filtration.

The dissection solution (DS) was composed of 8.0 ml of solution A, 0.5 ml of solution B and 2.0 ml foetal bovine serum (Flow Laboratories Ltd., Irvine, Ayrshire). Penicillin and streptomycin were added to give a final concentration of 100 IU/ml.

To prepare the culture medium, the dissection solution was enriched with tsetse pupal extract. Four or five surface sterilised pupae aged 8–12 days were crushed in 1.0 ml of DS. The mixture was centrifuged at 3500 rpm for 10 min and the clear supernatant fluid was decanted off as the culture medium.

Preparation of Tsetse Tissue Cultures

Newly deposited pupae of *G. morsitans* were kindly supplied by Dr. T. A. M. Nash, Tsetse Research Laboratory, Bristol. They were placed in pots of sterile sand and incubated at 25–27 C and with a relative humidity ranging between 65 and 80%.

Pupae of various ages and newly emerged adult flies served as sources of tissue for culture. The adult flies were immobilised in the refrigerator for 20 min so that they could be handled with ease. After washing in running cold water to remove extraneous sand particles, the pupae and flies were surface sterilised in 10% Roccal (Bayer Products Co.) for 10–15 min. Since the pupae tended to float in this liquid, they were immersed by gentle agitation of the flask every 4 or 5 min. They were washed several times in sterile physiological solution (Jones and Cunningham 1961) containing 100 IU/ml penicillin and streptomycin and 5 μg/ml Fungizone (E. R. Squibb and Sons Inc.). The washed pupae were transferred to a petri dish containing DS and with the aid of watchmaker's forceps, brain, alimentary tract, imaginal body wall, and reproductive organs were removed and placed in drops of culture medium.

The organs from pupae between 6 and 10 days old were cut into small fragments approximately 1 mm^3 with fine tungsten needles. Each tissue fragment was then placed in a small drop of culture medium spread as a thin film over a standard coverslip and left for 10 min to settle. The coverslips were inverted over depression chambers and sealed with a mixture of molten wax and vaseline. The chambers were then inverted and the cultures were incubated at 28 C as "sitting-drops" to encourage the cells to adhere to the coverslips.

Cultures of complete organs such as alimentary tract, reproductive organs, and mouth parts with attached salivary glands from pupae older than 18 days were set up in the same manner.

Preparation of Cultures of Trypanosomes

Mice were infected with *T. brucei* (TREU 667) and *T. congolense* (TREU 623). When the parasitaemia was high, blood was obtained aseptically from the tail for inoculation of the cultures. The blood was diluted with culture medium to give a concentration of about 1000 trypanosomes/mm^3. Fifteen–20 μl of the suspension of trypanosomes were placed on standard coverslips and one piece of tissue from freshly dissected pupae was added to each drop. The coverslips were inverted over depression chambers and the cultures set up as described. Trypanosome cultures were prepared in the same medium but in the

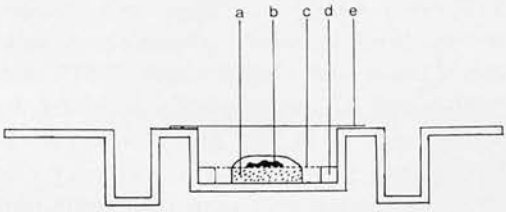

FIG. 1. Diagram of the slide tissue culture chamber showing (a) the trypanosome suspension, (b) the tsetse pupal alimentary tract, (c) semipermeable membrane, (d) silicone O-ring, and (e) coverslip.

absence of tsetse tissues. All cultures were incubated at 28 C.

Cultivation of Trypanosomes in Tissue Culture Chambers

Slide tissue culture chambers (Sterilin Ltd., Richmond, Surrey) as shown in Fig. 1 were used for observing the growth of *T. brucei* when separated from pupal alimentary tract by a semipermeable membrane. Bloodstream forms of *T. brucei* were mixed with culture medium and a drop of about 15 μl of trypanosome suspension was added to the deeper well of the chamber (a). A silicone rubber O-ring for a Millipore Swinnex 13 filter (d) was used to support the sterile disk of visking dialysis tubing (c) which was placed over the trypanosome suspension. One complete alimentary tract (b) from a pupa older than 23 days was carefully placed on the dialysis membrane immediately above the drop containing the trypanosomes. The tsetse tissue was kept moist in a drop of 5 μl of culture medium. The rim of the well was smeared with vaseline and the chamber was sealed with a standard coverslip (e). The perfusion channels were sealed with molten wax and the culture chamber was placed in a petri dish containing cotton wool saturated with sterile distilled water to avoid evaporation during incubation.

When these cultures were observed under the microscope any condensation which appeared on the coverslip was re-

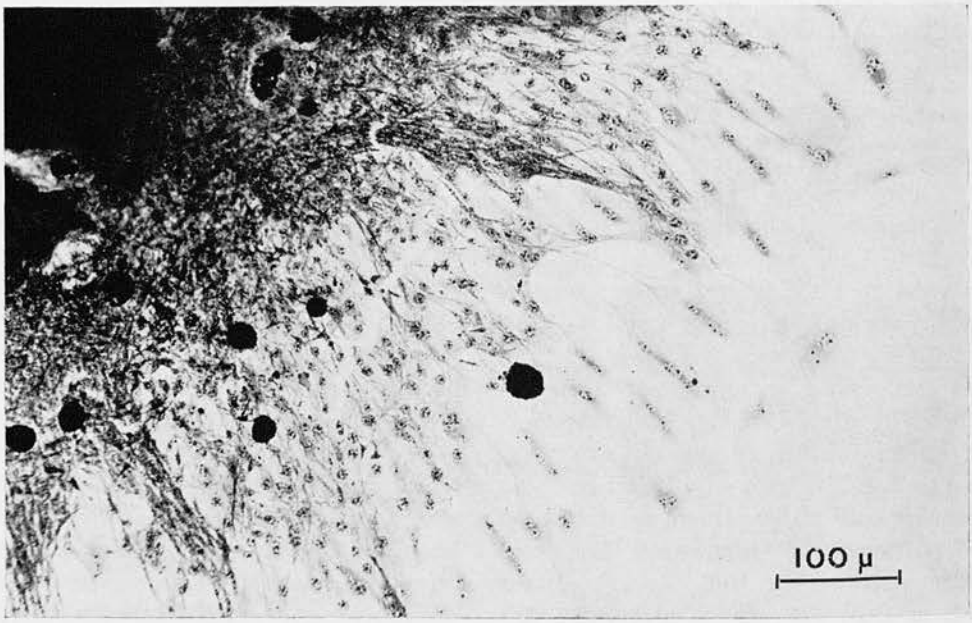

FIG. 2. Outgrowth of cells from an explant of proventriculus of a tsetse pupa aged 9 days; 6-day-old culture stained *in situ*.

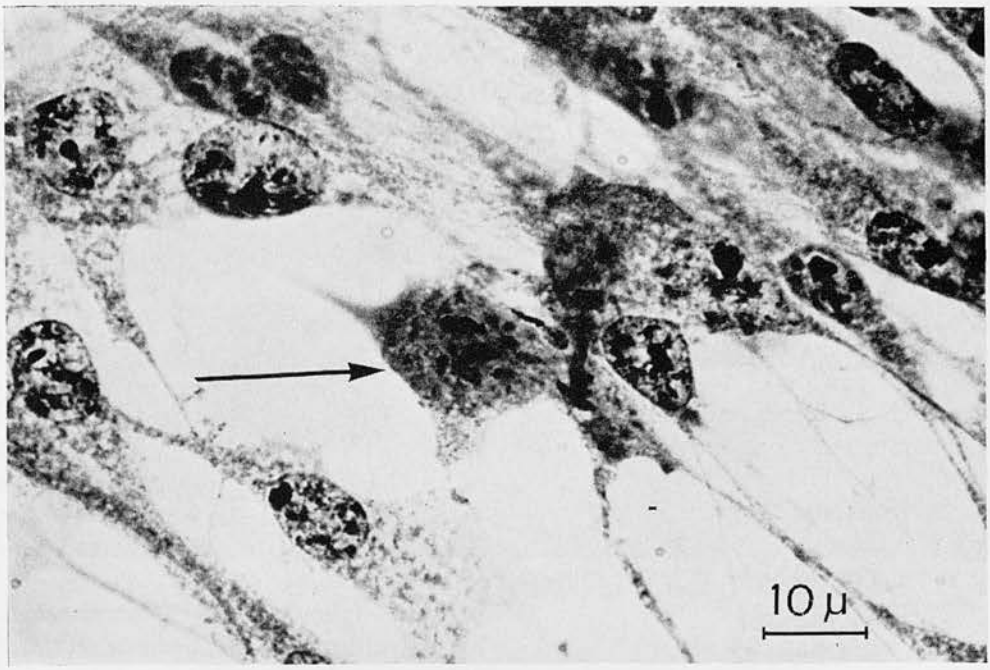

FIG. 3. Mitosis in the proventricular cells; 7-day-old culture.

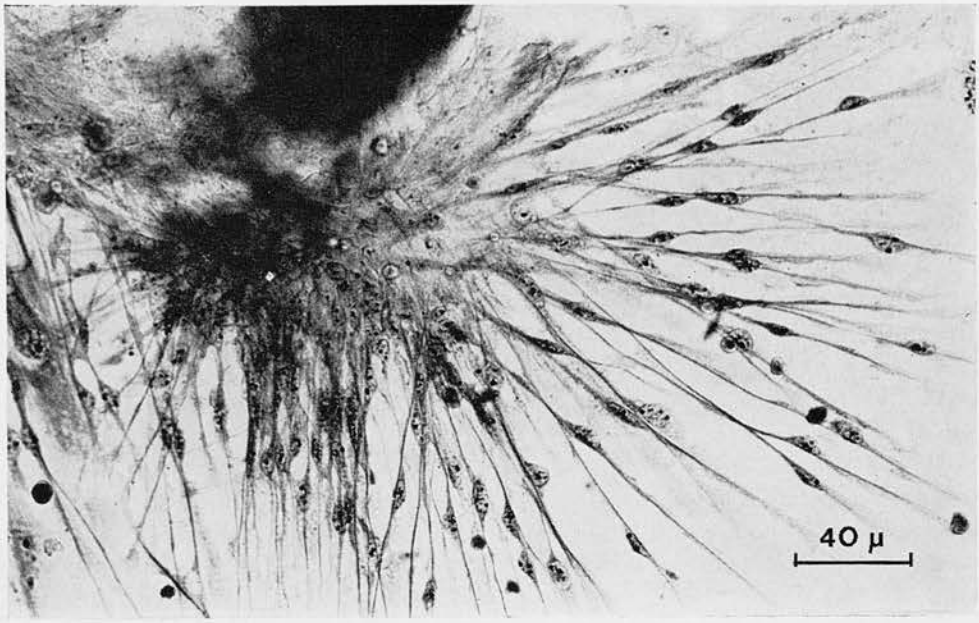

FIG. 4. Initial cell migration from an explant of imaginal body wall of an 8-day-old pupa; 3-day-old culture.

moved by sliding a hot needle over its surface.

Measurement of Growth of Trypanosomes

To measure the growth of the trypanosomes, cultures were prepared in replicate. Each day two or three cultures were examined and discarded. The medium was withdrawn into a capillary pipette and the trypanosomes washed off the coverslip with 9 vol of physiological saline. After thorough mixing a sample was introduced into a Neubauer haemocytometer and the number of trypanosomes per mm^3 was counted. Each experiment was repeated on two or three occasions. The graphs represent the arithmetic mean and standard deviation of four or six counts. Only motile trypanosomes were counted since it was assumed that only these were viable.

Results

Tsetse Tissue Culture

Cell migration from explants of various tissues of 6- to 10-day-old pupae began on about the third day after the cultures had been prepared. The type of growth observed varied with the different tissue. In cultures of the proventriculus, large fibroblast-like cells had spread some considerable distance from the explant by the sixth day of cultivation (Fig. 2), and mitoses were evident in stained preparations (Fig. 3).

Pieces of brain of 6-day-old pupae produced extensive cellular outgrowths after 4 days *in vitro*. The network of cells increased in area until the ninth day when no further outgrowth occurred. The lissamine green viability test (Goldacre and Sylven 1959) indicated that the elongated cells with their delicate cytoplasm containing fine refractile granules remained healthy for 15–20 days with two changes of medium.

When explants of imaginal body wall of 7–9-day-old pupae had been in culture for 3 days, initial outgrowths consisting of long narrow cells with central nuclei were observed (Fig. 4). These cells remained healthy for 12 days but mitosis was not seen.

Fig. 5. Vesicles extruded from a fragment of midgut of a 10-day-old pupa; 4-day-old culture.

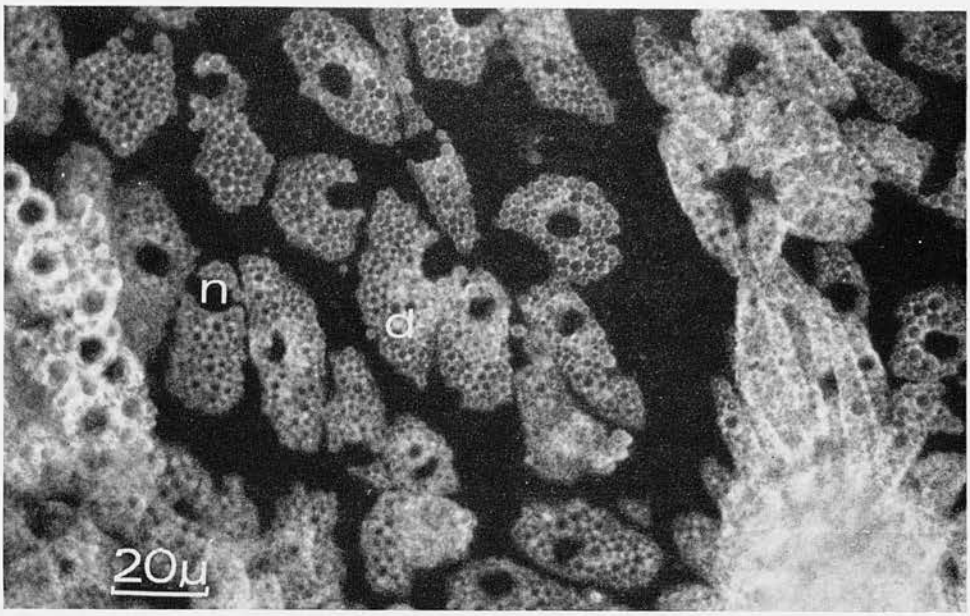

FIG. 6. Detail of Fig. 5 showing the cells of the walls of the vesicles. The nuclei (n) are surrounded by clusters of lipid droplets (d).

Fragments of midgut from pupae aged 8–10 days, however, displayed a different type of behaviour *in vitro*. After 2 or 3 days, large hollow vesicles were extruded from the edge of the explants. These vesicles continued to increase in size until they had dwarfed the original explant tissue (Fig. 5). In dark ground phase-contrast microscopy the nuclei of the cells were seen to be surrounded by dense clusters of droplets (Fig. 6). Staining with fat red 7B or oil red O demonstrated their lipid nature. After 10 days the vesicles began to contract rhythmically and this activity was maintained for periods of up to 21 days.

Complete alimentary tract from pupae older than 18 days showed active peristalsis of the crop, midgut, malpighian tubules, and hindgut regions for more than 3 weeks when the culture medium was replaced every 5 or 6 days. Salivary glands from pupae of the same age and from newly emerged adult flies displayed rhythmic contractions for only 7 days. When testes of pupae aged 25–27 days had been in culture for 2 weeks innumerable highly motile free spermatozoa were seen.

Growth of Trypanosomes in Tsetse Tissue Cultures

The medium alone, used to culture the tsetse tissues, did not support any growth of *T. brucei*. There was a decline in the number of living trypanosomes until the fourth day when they had completely disappeared. When entire alimentary tract from pupae aged about 22 days was included in the cultures there was a marked increase in the number of parasites. There was always a lag phase of about 24 hr followed by a period of exponential growth during which the number of organisms increased about 30 fold (Fig. 7). The numbers remained at this level for a further 4 days and then decreased until about Day 14 when no living trypanosomes were observed. When the trypanosomes were subcultured into culture drops containing freshly dissected alimentary tract, an exactly similar pattern of growth occurred.

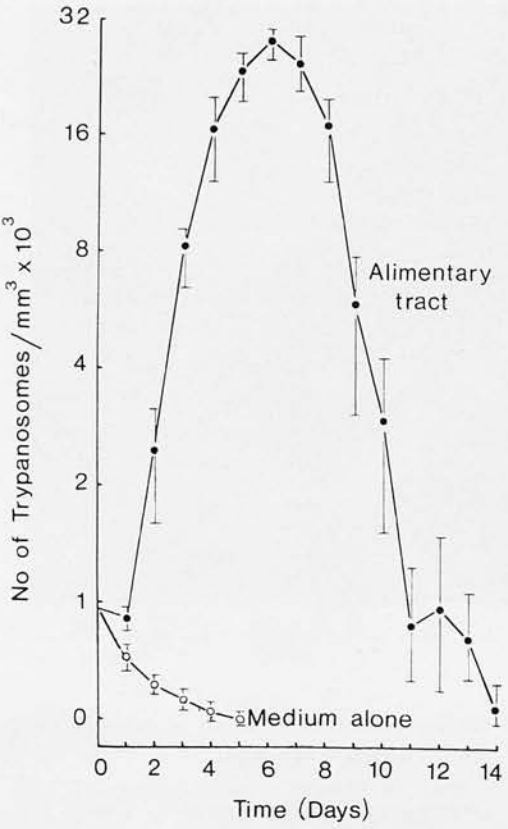

FIG. 7. The growth of *Trypanosoma brucei* in cultures of alimentary tract from 22-day-old pupae, and in culture medium alone. Means and standard deviations of four experiments.

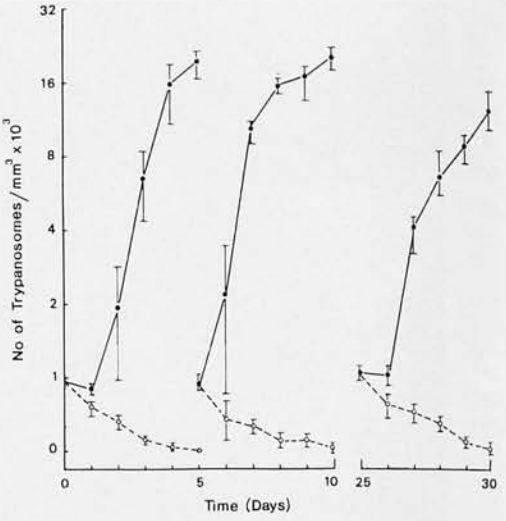

FIG. 8. Growth of *Trypanosoma brucei* when serially passaged every fifth day. (—) in the presence of pupal alimentary tract; (– – –) in culture medium alone. Means and standard deviations of four experiments.

Five such serial transfers were carried out covering a period of 30 days (Fig. 8). *T. congolense* was also inoculated into cultures containing pupal alimentary tract in the same manner. The result was identical with that obtained with *T. brucei*.

In the next series of experiments the effect of different organs on the growth of *T. brucei* was tested. The influence of the type of tissue on the growth of trypanosomes is indicated in Fig. 9. After 5 days the number of parasites was highest in cultures containing alimentary tract and lowest in those with body wall tissue.

It was thought that the state of development of pupal organs might affect their

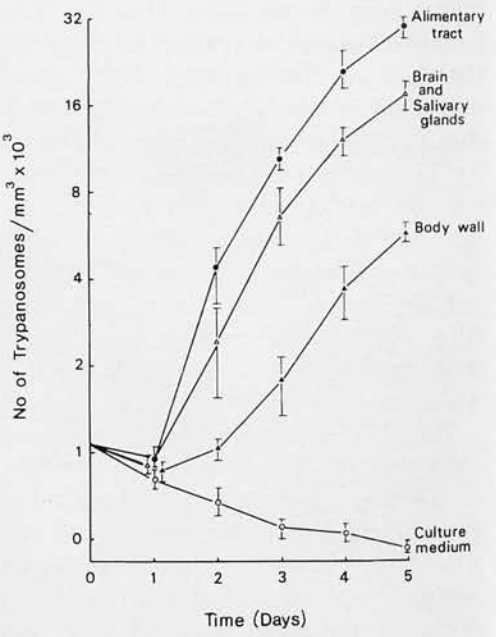

FIG. 9. Growth of *Trypanosoma brucei* in cultures of different tissues of pupae aged 25–27 days. Means and standard deviations of six experiments.

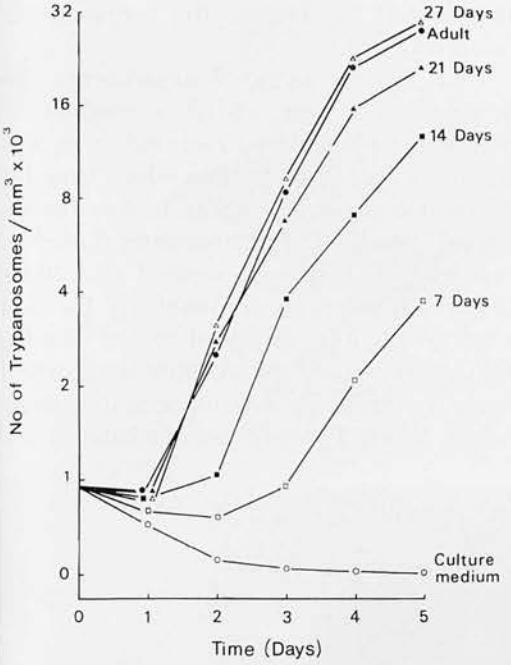

FIG. 10. Growth of *Trypanosoma brucei* in cultures containing alimentary tract from pupae of different ages. Means and standard deviations of six experiments.

ability to promote growth of the trypanosomes. Hence, *T. brucei* was introduced into cultures containing alimentary tract from pupae of different ages. As shown in

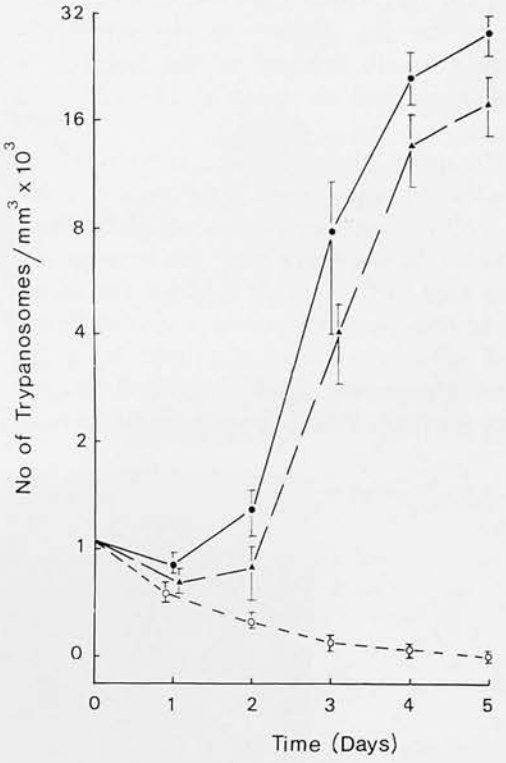

FIG. 11. Growth of *Trypanosoma brucei* in direct contact with pupal alimentary tract (—); in slide tissue culture chambers (– – –); in the absence of tsetse tissue (- - - -). Means and standard deviations of four experiments.

TABLE I

The Growth of Trypanosoma brucei in Cultures of Tsetse Pupal Alimentary Tract of Different Ages

Age of pupal alimentary tract (days)	Mean and standard deviation (SD) of No. of trypanosomes/mm³									
	Time (days)									
	1		2		3		4		5	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
7	745	130	642	182	983	282	2137	1163	3900	2145
14	838	109	1060	224	3891	458	7409	876	13362	796
21	876	85	2968	97	7046	892	15955	4323	22048	3229
27	847	81	3310	557	9165	484	23901	1603	29150	1423
Adult	857	69	2660	493	8669	816	22058	3240	29036	1967
Medium alone	516	146	177	45	60	27	6.6	7.5	0.8	2.0

Initial inoculum ranged between 875 and 1025 trypanosomes/mm³

Fig. 10 and Table I with undifferentiated tissue the lag phase was prolonged and only a small increase in the number of organisms had occurred at the end of 5 days.

Complete alimentary tracts from 22- to 25-day-old pupae were homogenised in 100 μl culture medium in an all-glass tissue grinder. In one experiment this homogenate was used as the culture medium and in another test the homogenate was centrifuged and after removal of the lipid layer the clear supernatant fluid constituted the culture medium. When these media were inoculated with *T. brucei*, the trypanosomes failed to grow.

In a further series of experiments, the trypanosomes were added to medium in which the alimentary tract had been cultured for 3 or 4 days, after which time the tissue was removed. Again, in this "conditioned" medium, the organisms failed to multiply. It, therefore, seemed that living tissue was required, consequently the next experiments were designed to see whether the trypanosomes would grow if separated from the tissue by a semipermeable membrane. When *T. brucei* was inoculated into

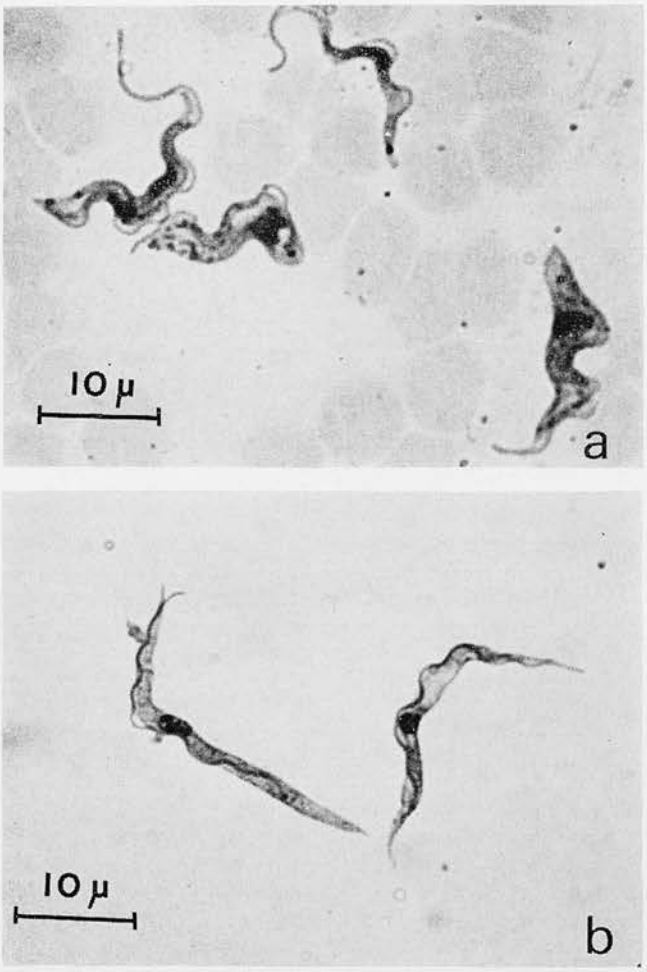

FIG. 12. (a) Mouse bloodstream forms of *Trypanosoma brucei* and (b) tsetse tissue culture forms of *T. brucei;* 6-day-old culture.

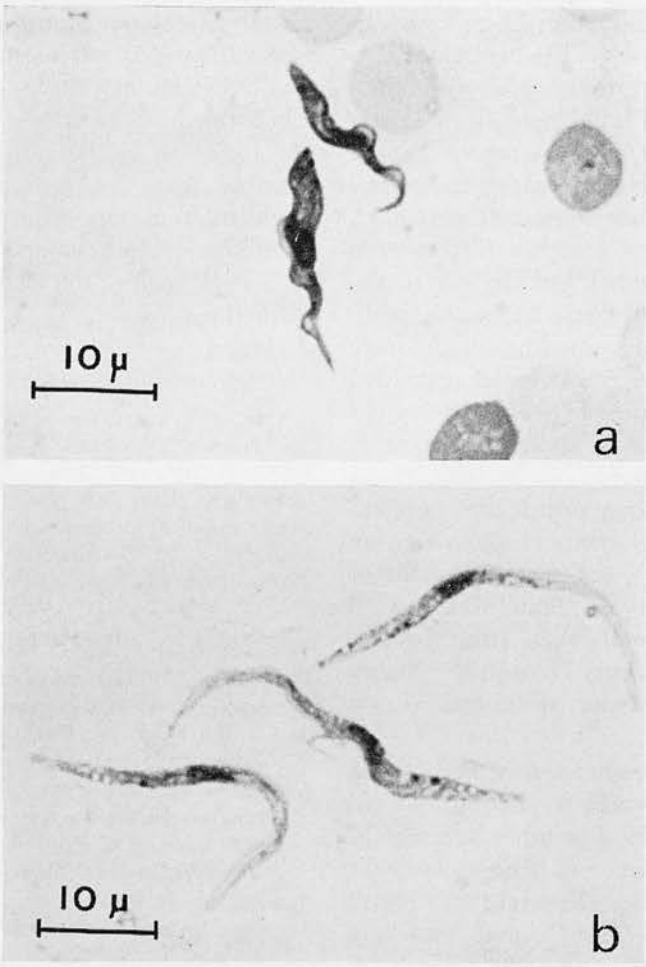

FIG. 13. (a) Mouse bloodstream forms of *Trypanosoma congolense* and (b) tsetse tissue culture forms of *T. congolense;* 10-day-old culture.

Sterilin slide tissue culture chambers the growth pattern was similar to that of control cultures where the organisms were in direct contact with the tissue (Fig. 11).

In all the cultures where multiplication of the trypanosomes occurred it was accompanied by morphological transformation; this took place in 3 or 4 days. Bloodstream forms (Figs. 12a and 13a) changed into long slender forms (Figs. 12b and 13b) similar to those seen in the midgut of the tsetse fly. The trypanosomes were usually evenly dispersed throughout the culture drops, but when separated from the tissue by a membrane, they tended to be concentrated in the neighborhood of the overlying tissue.

DISCUSSION

In modified Trager's medium, peristalsis was displayed by complete organs of *G. morsitans* pupae older than 18 days. This phenomenon is characteristic of many insect organs *in vitro*. In the same medium, cells migrated from explants of various pupal tissues. Mitoses occurred in cellular outgrowths from pieces of proventriculus which suggests that this tissue might be

suitable for the production of cell suspensions of monolayers. The cellular outgrowths resembled those produced from tissue explants of *G. palpalis* (Trager 1959). The midgut of *G. morsitans* formed large vesicles *in vitro* in contrast to the cell sheets described in similar cultures of *G. palpalis*. The reason for this difference in behaviour is not known but the age of the tissue, the species of tsetse fly, or the modification of the culture medium might have contributed to it. The vesicles extruded from midgut tissue of 8- to 10-day-old pupae were similar to those described in cultures of leafhopper embryonic tissue (Mitsuhashi and Maramorosh 1964), regenerating leg of the nymphal cockroach *in vitro* (Marks 1968), and tissue explants of lepidopteran embryos (Sohi 1967). Cell lines have been established from vesicles in cultures of larval mosquito tissues (Singh 1967; Schneider 1969; and Varma and Pudney 1969).

Media which have been described for the cultivation of salivarian trypanosomes have contained either blood or other living cells. Growth in the presence of mammalian cells has been reported by Demarchi and Nicoli (1960), Le Page (1967) and Hawking (1971) and in chick embryo fibroblasts by Fromentin (1961). The blood-enriched media are mostly diphasic (Tobie 1964) and the quantity of blood required is relatively large. More recently, liquid media in which the amount of blood is reduced has been described by Pittam (1970) and Dar (1971). All these media however, including those reported in the present studies are complex and undefined. In the conditions described in the present work, *T. brucei* and *T. congolense* multiplied and changed into forms morphologically similar to those found in the midgut of tsetse flies. The magnitude of the multiplication was measured and in the conditions described a 30 fold increase in number was achieved. The factor or factors supplied by the living tsetse tissues *in vitro* would appear to be small molecules since growth occurred when the organisms were separated from the tissue by dialysis tubing. It is, thus, possible that the system now described might offer an opportunity for studying the nutritional requirements of the trypanosomes and lead to the development of more defined media.

Acknowledgments

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Amino Acid Analyses of Haemolymph of *Glossina morsitans morsitans* (Westwood)

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Amino Acid Analyses of Haemolymph of *Glossina morsitans morsitans* (Westwood)

ISABEL CUNNINGHAM [1] and JOHN S. SLATER [2]

Abstract

Haemolymph from pupae and adult *G. morsitans* was analysed for its content of free amino acids. Of the twenty-one amino acids identified proline occurred in the greatest concentration in the 3 stages tested. Substantial amounts of taurine, glutamine, glutamic acid, alanine, tyrosine and arginine were also measured. There was considerable variation in the content of amino acids in the 3 stages of development of the tsetse fly.

Introduction

Insect haemolymph characteristically contains high levels of free amino acids (LEVENBOOK, 1950; FLORKIN, 1968), but their relative concentrations are subject to variation. Virtually any amino acid may constitute a major component in the haemolymph of a species at some stage of its life history and be almost lacking in another species or in another stage of the same species.

Knowledge of the chemical compositions of haemolymph has served as a valuable guide for the design of several successful culture media which have been used to grow the cells of a variety of insects and other arthropods (WYATT, 1956; SCHNEIDER, 1964; REHACEK & BRZOSTOWSKI, 1969; SCHNEIDER, 1971), but few attempts have been made to cultivate the tissues and cells of tsetse flies. TRAGER (1959) developed a culture medium which supported extensive cell migration from pieces of pupal tissues of *Glossina palpalis*, but NICOLI & VATTIER (1964) were less successful in their attempts to cultivate tissues of *G. fuscipes* in the same medium.

The present study was undertaken in the hope that an analysis of the free amino acids of tsetse haemolymph might therefore provide a guide for the design of a more consistent culture medium for the maintenance and growth of tsetse tissues than that currently in use in which lactalbumin hydrolysate is the main source of amino acids (CUNNINGHAM, 1973).

Materials and Methods

Newly deposited *G. morsitans* pupae supplied by Dr. T. A. M. Nash, Tsetse Research Laboratory, Langford, Bristol, were placed in pots of sterile sand and incubated at 25 °C and at a relative humidity between 65–80 %. Pupae, pre-emerged flies and 2-days old flies were used as sources of haemolymph.

During development of *Glossina* species larval/pupal and pupal/adult apolyses [3] take place and at approximately 16 days after larviposition the dark rigid

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[3] Apolysis = detachment of the epidermal cells from the old cuticle.

puparium contains a pharate[4] fly (SAUNDERS, 1970; HINTON, 1971). In this study the term 10 days old pupae refers to the contents of the puparia 10 days after larviposition.

Collection of haemolymph

Pupal haemolymph – Pupae of 10 days old were washed in sterile distilled water to remove extraneous sand particles. They were allowed to dry and were placed in a refrigerator at 4 °C for 30 minutes. With the aid of watchmakers' forceps, a small fracture was made at the anterior end of the puparium. A capillary tube was inserted through the ruptured puparium into the pupal haemocoel. By capillarity, haemolymph and fat body entered the tube; care was taken to avoid collecting the gut. The contents were expelled onto a few crystals of phenylthiourea in a small Petri dish held over an ice bath. Precooling the pupae and adding phenylthiourea prevented melanization of the haemolymph, which results from oxidation of tyrosine in the presence of an active polyphenol-polyphenoloxidase system (DUBOIS & ERWAY, 1946). The haemolymph of 10 pupae was pooled and centrifuged in a haematocrit tube at 10,000 r.p.m. for 10 minutes. The supernatant fluid, excluding the lipid layer, was transferred to a small tube and stored at –20 °C until used.

Haemolymph of pre-emerged flies – Pupae aged 27 days were washed and precooled at 4 °C for 30 minutes. The anterior end of the puparium was removed with fine forceps and flies at the point of emergence everted the ptilinum. When the ptilinum was fully extended it was cut with iridectomy scissors and the haemolymph was collected in a haematocrit tube. Each tsetse fly yielded approximately 6 μlitres of haemolymph which was treated by the method described above. Haemocytes were removed by centrifugation at 2,000 r.p.m. for 5 minutes and the haemolymph was stored at –20 °C.

Haemolymph of adult flies, 2 days after emergence – Unfed flies were immobilised in the refrigerator at 4 °C for 30 minutes. The haemolymph was collected by amputating the posterior leg at the femur. A capillary tube was held at the stump end and gentle pressure on the thorax of the fly expelled the haemolymph into the tube. A maximum of 2 μlitres could be obtained from one fly. The haemolymph of 15–20 flies was treated as described above and stored at –20 °C.

Amino acid analyses – Immediately before analyses, pooled samples of 10–20 μlitres of haemolymph were placed in small tubes and the volume adjusted to 2.5 ml with 0.1 *N* HCl containing 10 % sucrose and 0.25 μmole norleucine. One ml samples of this solution were analysed for free amino acid content using a Technicon Nc-1 Amino Acid Analyser.

Results and Discussion

The results of the analyses of the free amino acids in haemolymph of pupal and adult tsetse flies are illustrated in Fig. 1 and Table 1. The total concentration of amino acids increased from 1,756 mg/100 ml in the pupal haemolymph to 2,021 mg/100 ml in the pre-emerged flies and decreased to 1,486 mg/100 ml in flies which were 2 days old. These values were within the range found in other insect species (100–2,000 mg/100 ml) and were between 30 and 40 times higher than those recorded for vertebrate plasma (40–60 mg/100 ml) (BURSELL, 1970).

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[4] Pharate = that part of a new instar of an arthropod which is enveloped by the cuticle of the previous stage.

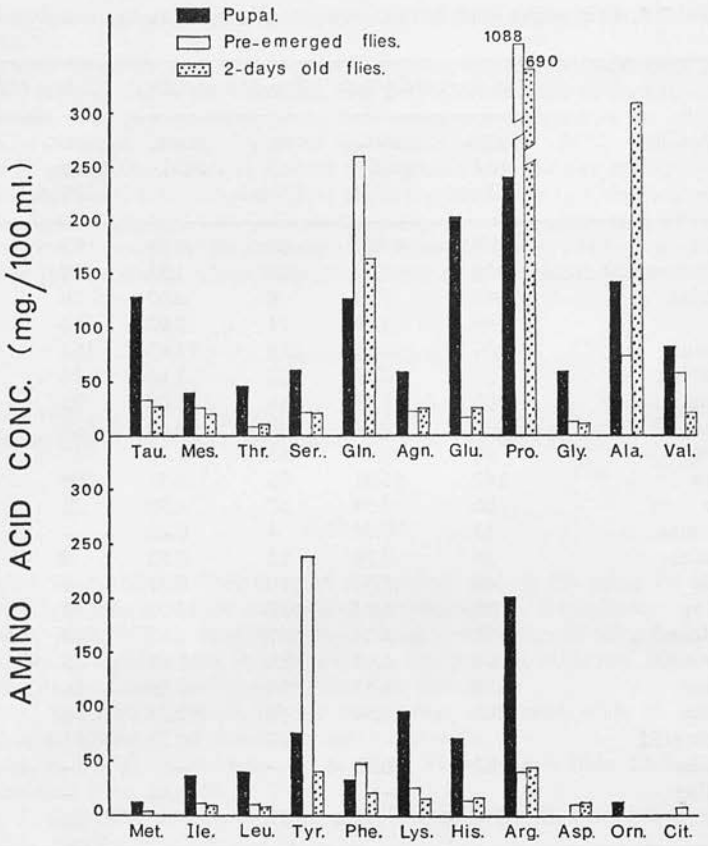

Fig. 1. Amino acid analyses of haemolymph of 10 days old pupae and flies *(G. morsitans).* Tau-taurine; Mes-methionine sulphoxide; Thr-threonine; Ser-serine; Gln-glutamine; Agn-asparagine; Glu-glutamic acid; Pro-proline; Gly-glycine; Ala-alanine; Val-valine; Met-methionine; Ile-Isoleucine; Leu-leucine; Tyr-tyrosine; Phe-phenylalanine; Lys-lysine; His-histidine; Arg-arginine; Asp-aspartic acid; Orn-ornithine; Cit-citrulline.

These was, however, considerable variation in the levels of individual amino acids between the 3 stages. This was particularly marked for taurine, glutamine, glutamic acid, proline, tyrosine, lysine, histidine and arginine. Most of the amino acids occurred in greater concentrations in the pupal haemolymph than in the haemolymph of flies. Exceptions to this were glutamine, proline, alanine, tyrosine, phenylalanine and aspartic acid. The last of these was not detected in the pupal blood. In the pre-emerged and 2-days old flies, the levels of most of the amino acids were similar but there was a marked decrease in the amount of glutamine, proline, valine and tyrosine in the older flies. Variation in the levels of amino acids between the 3 stages was not unexpected since significant differences in amino acid content are known to occur during the life history of a single insect species (FLORKIN & JEUNIAUX, 1964; CHEN, 1966). Some of the observed variations were probably associated with short-term fluctuations in the concentration of particular amino acids concerned with different metabolic activities.

Table 1. Free amino acids in haemolymph of tsetse *Glossina morsitans*

Amino acids	10 days old pupae		Pre-emerged flies		2 days old flies	
	conc. mg/ 100 ml	conc. mM	conc. mg/ 100 ml	conc. mM	conc. mg/ 100 ml	conc. mM
Taurine	127.2	10.17	33	2.67	27	2.15
Methionine sulphoxide	38	2.31	25	1.53	20	1.22
Threonine	45	3.74	8	0.67	10	0.86
Serine	60	5.74	21	2.03	20	1.93
Glutamine	126	8.61	259	17.65	164	11.25
Asparagine	57	4.31	22	1.66	24	1.79
Glutamic acid	203	13.79	16	1.08	25	1.70
Proline	239	10.74	1,088	94.60	690	60.04
Glycine	59	7.93	13	1.72	12	1.64
Alanine	142	15.91	73	8.15	309	34.68
Valine	86	7.34	57	4.90	21	1.78
Methionine	12	0.79	4	0.28	–	–
Isoleucine	36	2.78	12	0.92	9	0.67
Leucine	40	3.06	10	0.77	9	0.66
Tyrosine	76	4.22	237	13.07	40	2.21
Phenylalanine	33	2.03	47	2.84	20	1.19
Lysine	94	6.45	25	1.68	15	1.04
Histidine	70	4.52	14	0.90	16	1.01
Arginine	201	11.54	40	2.28	44	2.54
Aspartic acid	–	–	10	0.78	11	0.86
Ornithine	12	0.93	–	–	–	–
Citrulline	–	–	7	0.42	–	–
Total	1,756		2,021		1,486	

A striking feature was the high concentration of proline particularly in the haemolymph of the pre-emerged flies in which it reached a level of 1,088 mg/ 100 ml. Proline plays an active part in general protein metabolism and is a major component in the protein of insect cuticle (HACKMAN, 1953). The investigations of BURSELL (1963) on the amino acid content of the musculature of *G. morsitans* during the hunger cycle revealed large amounts of proline in the resting flies but the level diminished during flight. The decrease in the concentration of proline in the 2-days old flies in the present studies might have been associated with either flight metabolism or cuticle formation or both. BALOGUN (1969, 1971) recorded relatively low concentrations of proline in the amino acid analysis of extracts of homogenised whole *G. palpalis* flies using the technique of paper partition chromatography. The notable increase in the level of alanine in the 2-days old flies confirms the findings of BURSELL (1963) in which the alanine content of the musculature of the thorax of tsetse flies increased dramatically after flight. Large amounts of alanine were also identified on chromatograms of haemolymph of adult *G. pallidipes* (KNIGHT, 1961) and in extracts of whole flies *G. palpalis* (BALOGUN, 1971). The fluctuation in the quantity of tyrosine could be explained by the general accumulation of tyrosine in the blood during adult development in preparation for the final stages of sclerotization and tanning of the cuticle which takes place at emergence of adult flies. Biochemical studies of this compound

indicate that it enters important metabolic activities of most insects at metamorphosis.

Certain amino acids such as threonine, glycine, methionine, isoleucine, leucine, aspartic acid, ornithine and citrulline were poorly represented in the haemolymph of the flies. A large proportion of the methionine was detected as the sulphoxide which is produced during storage of the haemolymph at –20 °C. The variations in the other amino acid levels are not fully understood and any attempt at interpretation with our knowledge of tsetse physiology would be speculative at present.

The figures obtained may indicate the range of amino acids desirable for the formulation of media for the cultivation of tsetse fly tissues, and they may also be of value for the design of media for the growth of tsetse-borne parasites *in vitro*.

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